THE PLASTICITY OF PANCREATIC CELLS

by

Yue Julia Wang

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Abstract

The pancreas is a gland with both exocrine and endocrine roles. It is both involved in digesting food and secreting the hormones that regulate blood glucose. Two important diseases related to these two components of the pancreas are pancreatic cancer and diabetes.

The fundamental problem for diabetes is the deficiency of insulin-secreting β cells. One strategy to reverse the diabetic condition is to supplement patients with more β cells, either by transplantation of *in vitro* derived β cells or by stimulating endogenous progenitor cells to differentiate into mature β cells. In Chapter 2, I describe the study I carried out in zebrafish to lineage trace a *ptf1a*-expressing cell population. Insights we obtained from studying the endogenous pancreatic developmental mechanism will hopefully benefit us in deriving more efficient method to generate β cells in the future. In Chapter 3, I present the work on characterizing an Aldefluor positive, Sca-1 positive progenitor population in the adult mouse pancreas. The motivation of this study is that this progenitor population may represent a cellular source that could be readily exploited to generate new β cells.

Another important disease of the pancreas is pancreatic cancer. Rigorous lineage tracing studies have shown that acinar cells are the cellular origins for pancreatic ductal adenocarcinoma, the predominant type of pancreatic cancer. In Chapter 4, I depict the work on the role of Dicer, the master regulator of miRNA pathway, in the pancreatic acinar tissue homeostasis and during the initiation of pancreatic cancer. In Chapter 5, I outline our

efforts in characterization the role of one particular miRNA cluster, mir-216b-217, during the processes of pancreatic acinar cell-fate maintenance and pathophysiological changes of pancreatic cancer.

Together, my thesis work provides new insight into the plasticity of pancreatic cells and provide the scientific field with novel tools for further investigating the mechanisms of pancreatic development, diabetes and pancreatic cancer. It is hoped my efforts will also point to potential directions to derive clinical therapies.

Thesis readers:

Professor Steven D. Leach

Professor Michael J. Parsons

Professor Steven A. Farber

Dedication

This dissertation is dedicated to my loving and supportive parents, 高翔，王家明, and my always encouraging late grandmother, 谢惠芝。

Contents

List of Figures

Acknowledgements

I still vividly remember the first day when I came to Johns Hopkins, on Aug. 17th, 2007. It was the second day since my arrival in the U.S., after a 20 hours flight from China. Help and support from numerous people around me made me feel right at home.

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Chapter 1 Introduction

1.1 The cellular composition of the pancreas

The pancreas is a remarkable organ. It performs both endocrine and exocrine functions (Fig. 1.1). The exocrine component consists of two cell types: (1) acinar cells that secrete digestive enzymes, including proteases, amylases, lipases and nucleases; and (2) ductal cells that convey the enzymes to the intestine. The endocrine component consists of five cell types: the glucagon-secreting α cells, the insulin-secreting β cells, the somatostatin-secreting δ cells, the ghrelin-secreting ε cells, and the pancreatic polypeptide-secreting PP cells (Granata et al., 2010). Together, the endocrine cells secrete hormones into the bloodstream and these hormones regulate nutrient metabolism and glucose homeostasis.

Around 90% of the pancreas is composed of acinar cells. The acinar cells are grouped into units called acini. Acinar cells have polarized arrangement within the acini, with basally positioned nuclei and apically located granules of digestive enzyme or zymogen (Kern, 1993). Centroacinar cells are located at the junction of acini and ducts (Pour, 1994). Centroacinar cells connect with terminal intercalated ducts, which sequentially merge to intralobular ducts, interlobular ducts, and finally the main pancreatic duct. The pancreatic ductal cells secrete bicarbonate-rich fluid to facilitate transportation of the digestive enzymes secreted by acinar cells (Grapin-Botton, 2005; Reichert and Rustgi, 2011).

The endocrine cells are mainly grouped into the structure islets of Langerhans (Gomori, 1941; Granata et al., 2010). Scattered and isolated endocrine cells can also be

detected within the exocrine parenchyma. These endocrine cells are often associated with ducts (Bertelli and Bendayan, 2005; Bertelli et al., 2001).

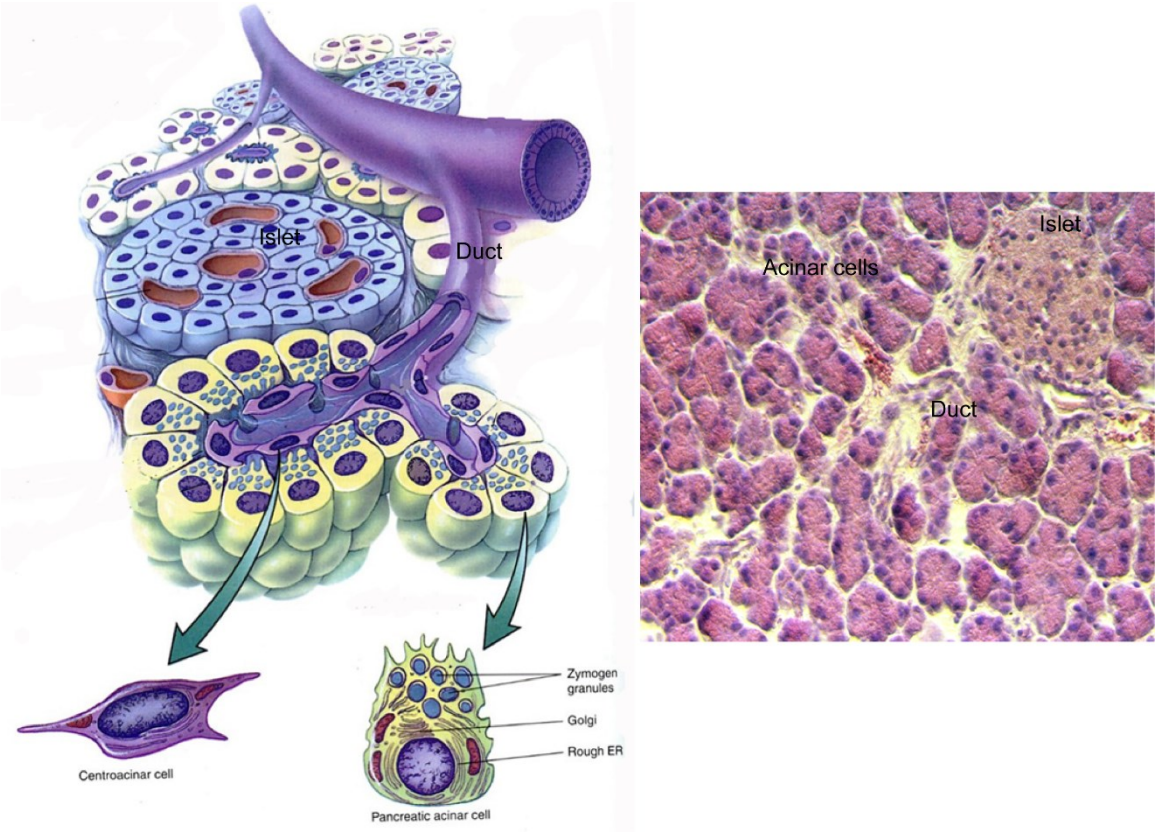
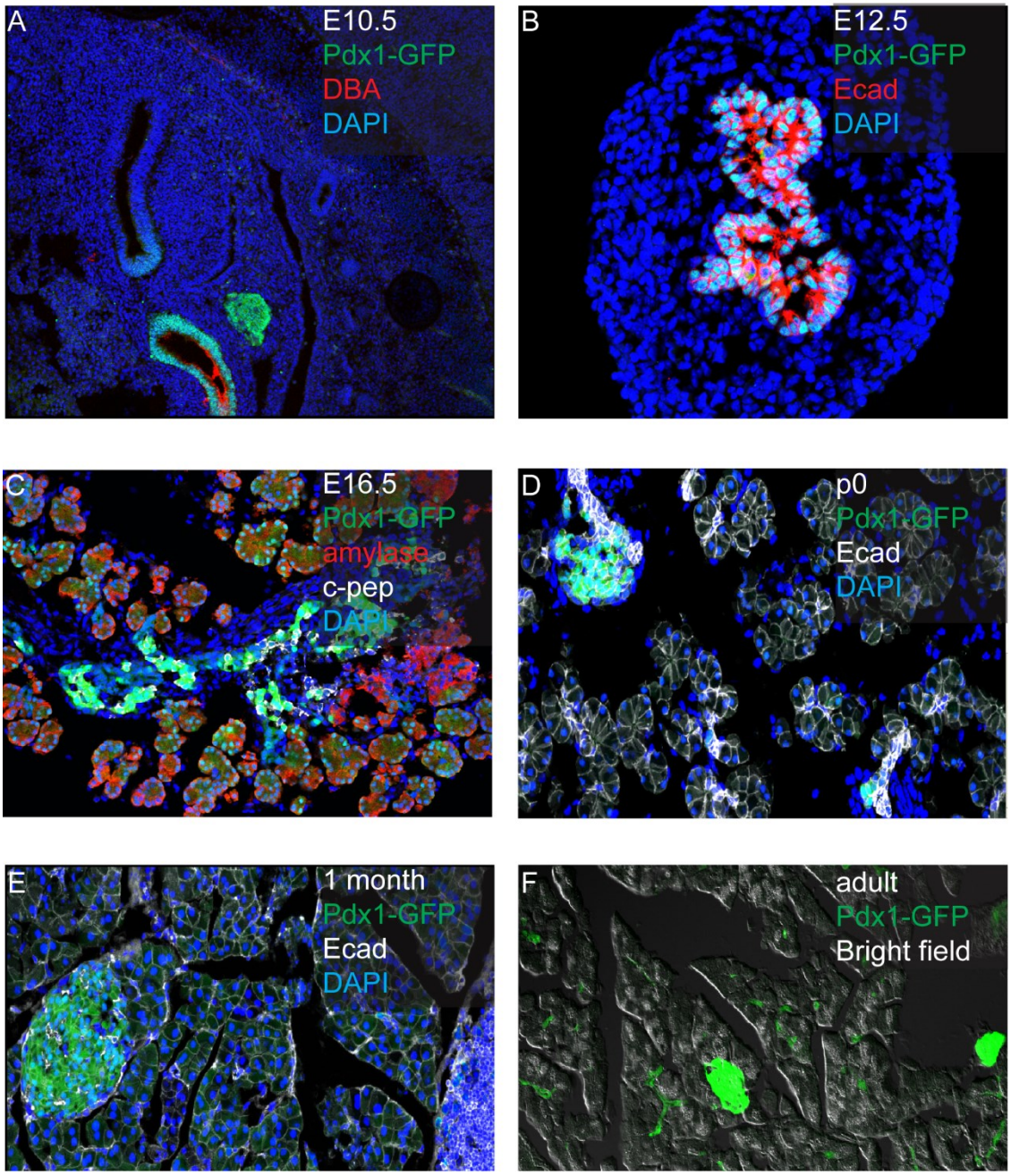


Figure 1.1: The cellular composition of the pancreas
(Reproduced from Yale University.)
Left, artistic rendition of the pancreas. Right, H&E showing a section of adult mouse pancreas.

1.2 Pancreas Development

In the mouse, pancreas development is first noticeable at around embryonic day 8.5~9.5 (E8.5~E9.5) as the thickenings of multilayered stratified epithelium in the foregut endoderm (Fig. 1.2A). The classical model of pancreas tubulogenesis involves epithelial budding and branching morphogenesis (Gittes, 2009; Slack, 1995). Recent studies,

however, have shown that epithelial polarization and intraepithelial lumen formation are required for the establishment of the ductal network in the mouse pancreas (Kesavan et al., 2009; Villasenor et al., 2010). Specifically, at E11.5, multiple microlumens are formed. Starting at E12.5, these lumens coalescence and rearrange into an interconnected tubular structure (Kesavan et al., 2009) (Fig. 1.2B). During the first wave of development, there is little cellular differentiation except the early appearance of glucagon positive cells (Herrera et al., 1991; Rall et al., 1973). Starting at E13.5, dramatic morphogenetic changes, the so-called "secondary transition", occur in the pancreas. The process is characterized by rapid proliferation and differentiation (Kemp et al., 1972; Pictet et al., 1972). Mature acinar cells are specified in the tips of the pancreatic bud, whereas endocrine cells and ductal cells are present in the trunk epithelial region (Fig. 1.2C) (Klinck et al., 2011; Kopp et al., 2011b; Pan et al., 2013; Schaffer et al., 2010; Solar et al., 2009; Zhou et al., 2007) (Fig. 1.2).


A
E10.5
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DAPI
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Pdx1-GFP
Ecad
DAPI
C
E16.5
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amylase
c-pep
DAPI
D
p0
Pdx1-GFP
Ecad
DAPI
E
1 month
Pdx1-GFP
Ecad
DAPI
F
adult
Pdx1-GFP
Bright field

Figure 1.2: Pancreas development.

Pancreata from *Pdx1-GFP* transgenic mice at different developmental time points were harvested and sectioned. Pdx1 is one of the earliest transcription factors to be expressed in the pancreatic field. It marks pancreatic epithelial components during development and is restricted to endocrine β cells and δ cells as well as ductal cells in the adult. In all the panels, signals from *Pdx1-GFP* transgene are shown in green. (A) E10.5. The dorsal buds are starting to develop. Notice that Pdx1 also expresses in the intestine. DBA (red) labels ductal cells (Reichert et al., 2013). DAPI, blue. (B) E12.5. In the dorsal buds, there is early onset of tubular morphogenesis. Ecad, red. DAPI, blue. (C) E16.5. The tip and truck domains are already evident. Differentiated acinar cells, marked by amylase (red) staining, accumulate in the tips. Endocrine populations, including insulin-secreting β cells (c-pep, white), are concentrated in the trunk domains. The endocrine population maintains high expression of Pdx1. DAPI, blue. (D) P0. Pancreatic morphology starts to resemble that of the adult, except that the acinar units are still small and relatively loosely attached. Ecad, white. DAPI, blue. (E) 1 month. Further organization of the pancreas. Ecad, white. DAPI, blue. (F) In the adult pancreas, fluorescent signals from the *Pdx1-GFP* transgene can be detected in the endocrine β and δ cells, as well as terminal ducts.

1.3 Ptf1a

A number of critical gene regulatory networds have been identified in the developing mouse pancreas. Pancreas transcription factor 1a (*Ptf1a*) is among the first genes to be expressed in the pancreas. Lineage tracing studies in the mouse show that *Ptf1a* is a marker of multipotent progenitor cells in the developing pancreas (Kawaguchi et al., 2002; Pan et al., 2013). Before E18.5, *Ptf1a*-expressing cells give rise to all pancreatic epithelial lineages including acinar, endocrine and ductal cells (Pan et al., 2013). *Ptf1a* is both necessary and sufficient for early pancreatic organogenesis. In *Ptf1a* null mice, the growth of dorsal and ventral pancreatic buds is severely retarded; there is complete lack of acinar cells and the endocrine cell numbers and distribution are altered (Burlison et al., 2008; Krapp et al., 1998). In addition, in the *Ptf1a*-deficient state, progenitor cells that should have been directed to the pancreatic fate are relocated to the duodenum or common bile duct instead (Burlison et al., 2008; Kawaguchi et al., 2002). One further piece of evidence, which establishes *Ptf1a* as the master regulator of pancreatic differentiation, is that ectopic expression of *Ptf1a*, together with another important transcription factor pancreatic duodenum homeobox 1 (*Pdx1*), is capable of converting duodenum to pancreas in *Xenopus* embryos (Afelik et al., 2006).

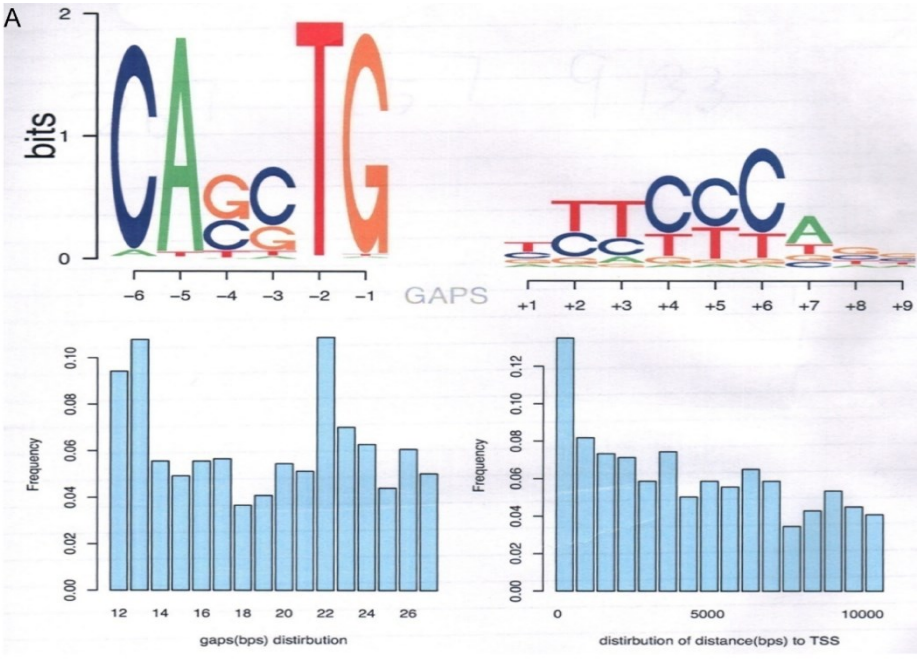
In the mature pancreas, the expression of *Ptf1a* is restricted to the acinar cells, and this is important for the maintaining of the pancreatic acinar cell fate. Antagonism of Ptf1a in the acinar cells induced the expression of endocrine-specific genes (Hesselson et al., 2011).

Ptf1a is the α-subunit of the trimeric protein complex PTF1. The other two components of the complex are the ubiquitous bHLH protein E2A and the Suppressor of Hairless (RBP-J) protein or its paralog RBP-JL (Beres et al., 2006). During the early stages of pancreatic development, Ptf1a interacts with RBP-J. This interaction is essential for the developmental functions of Ptf1a (Masui et al., 2007), one of which is to repress the alternative lineage program specified by high expression of Nkx6.1 (Schaffer et al., 2010). In mature acinar cells, however, Ptf1a switches its partner from RBP-J to RBP-JL, and together, they bind to promoters of acinar-specific genes such as those encoding digestive enzymes (Beres et al., 2006; Masui et al., 2010).

ChIP-seq results demonstrate that the PTF1 complex recognizes a DNA-binding sequence containing an E-box (CACCTG) and a TC-box (TTTCCCACG) spaced 1 or 2 helical DNA-turns apart (Fig. 1.3A) (Beres et al., 2006). A highly conserved 2.3 kb genomic region, between -15.6 kb and -13.4 kb relative to the transcriptional start site of *Ptf1a*, has been identified as an enhancer. The 5' enhancer contains two bipartite E-box and TC-box binding sites and has been shown to be bound by the PTF1 complex, thus establishing the autoregulatory loop of *Ptf1a* (Masui et al., 2008).

In humans, individuals with mutations in *PTF1A* present clinically with permanent neonatal diabetes mellitus and cerebellar agenesis (Al-Shammari et al., 2011; Hoveyda et al., 1999; Sellick et al., 2004; Tutak et al., 2009). The mutations in the *PTF1A* coding sequence uncovered to date are summarized in Fig. 1.3B. Recently, another cluster of mutations that are associated with pancreatic agenesis were found in the cis-regulatory region located 25kb downstream of *PTF1A* (Weedon et al., 2014) (Fig. 1.3C).

It is clear that zebrafish *ptf1a* plays an important role in the establishment of acinar cell fate, but its role in endocrine specification is still debatable. On the one hand, there is evidence that early endocrine lineage is independent of *ptf1a*. For example, it has been shown that *ptf1a* down-regulation by morpholinos impairs the development of exocrine pancreas, but the differentiation and organization of the principal islet is preserved (Lin et al., 2004). On the other hand, data has shown that a reduced level of *ptf1a* is more favorable for endocrine differentiation (Dong et al., 2008). One way to address the question of whether *ptf1a* is ever expressed (and thus likely required) in the endocrine progenitors is to carry out lineage-tracing experiments. In Chapter 2, I will present the lineage tracing results we obtained from the newly generated *ptf1a:creER*^{T2} fish line. Furthermore, the transparency of zebrafish larvae and their small size render it possible to carry out imaging studies on the whole embryos. As a result, I was able to simultaneously examine all the tissues that were labeled in the *ptf1a* lineage. Lineage analysis shows that in the wildtype pancreas, there are limited contribution of *ptf1a* lineage to pancreatic Notch-responsive-cells (PNCs) and endocrine fate. *ptf1a* lineage to some degree contributes to β-cell regeneration. However, in the *ptf1a* heterozygous mutant fish, a higher proportion of *ptf1a* lineage-labeled cells can be traced into PNCs and endocrine compartment. Further reduction of *ptf1a* dosage converts the ventrally derived pancreatic progenitor cells to gall bladder and other nonpancreatic cell fate. I shall discuss the different cellular differentiation roles of different threshold of *ptf1a* and the application in *in vitro* derivation of β cells.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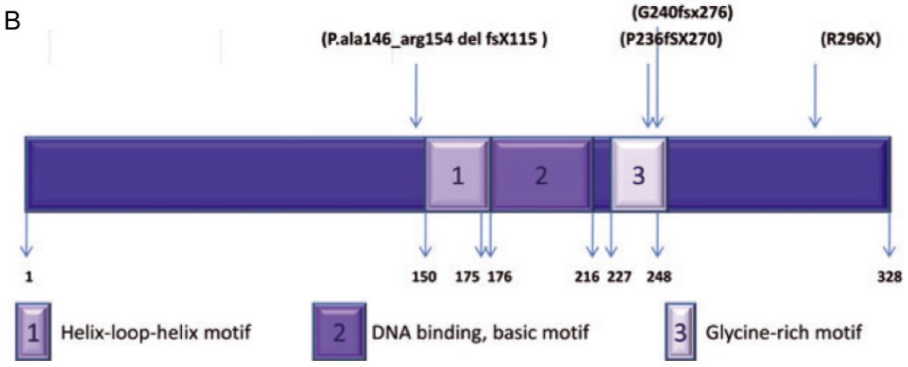
B
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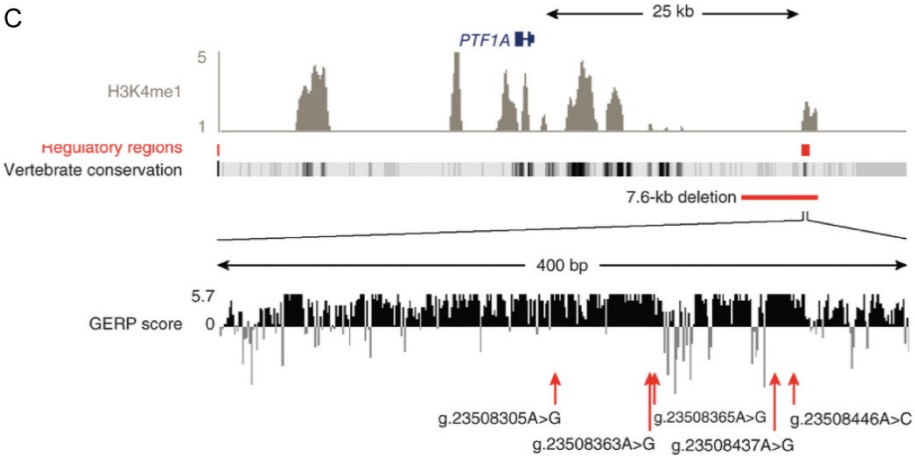
(G240fsx276)
(P236fSX270)
(R296X)
1
2
3
1 150 175 176 216 227 248 328
Helix-loop-helix motif
DNA binding, basic motif
Glycine-rich motif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GERP score
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g.23508363A>G
g.23508365A>G
g.23508437A>G
g.23508446A>C

Figure 1.3: PTF1 binding sequences and *PTF1A* mutations identified in human. (A) Sequence logos shows the canonical PTF1 binding sites, with an E-box (CACCTG) and a TC-box (TTTCCCACG) spaced 1 or 2 helical DNA-turns apart. Position weight matrix is based on published data from Masui, et al (Masui et al., 2010). Figure is adapted from Dr. Xiaogang Zhong, personal communication. (B) Summary of mutations that have been reported in *PTF1a* coding sequence. Figure is reprinted from Al-Shammari, et al (Al-Shammari et al., 2011). (C) Mutations in the upstream enhancer region of *PTF1a* that are associated with pancreatic agenesis. Figure is reprinted from Weedon, et al (Weedon et al., 2014).

1.4 The sources of new β cells

The most prevalent disease of the pancreas is diabetes mellitus. There are two major types of diabetes. In type 1 diabetes, the body's immune system attacks its own β cells (Gale, 2001). Type 2 diabetes is characterized by peripheral tissue insulin resistance and concomitant β-cell noncompensation (Butler et al., 2003). To correct the β-cell deficit of diabetes, there are two major areas that are under intense investigation. One is the directed differentiation of pluripotent stem cells *in vitro* and the other is the stimulation of regeneration or transdifferentiation of β cells *in vivo* (Fig. 1.3).

In vitro strategies that guide the differentiation of pluripotent stem cells into β-cell following developmental principles have been developed (D'Amour et al., 2006; Kroon et al., 2008). However, before β cells derived in a dish can be used for therapeutic purpose, there are major obstacles that have yet to be overcome. For example, the risks of tumorigenesis upon transplantation of stem cell-derived tissues have always been of concern (Carpenter et al., 2009). Furthermore, these β cells have exhibit a limited respond to glucose stimulus while in culture and require a final *in vivo* maturation step (D'Amour et al., 2006; Kroon et al., 2008). Recent studies have shown that these *in vitro* differentiated β cells more closely resemble embryonic β cells than adult mature β cells (Hrvatin et al., 2014; Xie et al., 2013).

Endogenously, the potential sources of new β-cells include preexisting β cells, acinar cells, α cells, ductal cells, liver cells, intestine cells, and pancreatic progenitor cells.

A pulse-chase experiment using *Ins-creER^{T2}; Z/AP* mice was designed to label mature β cells with alkaline phosphatase upon tamoxifen injection and chase them over

different periods of time. This experiment demonstrated that the percentage of labeled β cells is stable in adult life and during β-cell regeneration upon pancreatectomy. No dilution of label was observed. This observation implies that self-duplication of pre-existing β cells is the source of new β cells (Dor et al., 2004). However, this experiment did not exclude the possibility of the existence of progenitor cells, due to the variable labelling efficiency of the inducible creER^{T2} system (Guo et al., 2002; Hebert and McConnell, 2000; Kawaguchi et al., 2011).

Another approach to generate new β-cells is by transdifferentiation. Acinar cells are one of the promising sources. The forced expression of three transcription factors, Pdx1, MafA and Ngn3 (PMN), is sufficient to convert acinar cells into β cells (Zhou et al., 2008). Suppression of Ptf1a activity induces acinar-to-endocrine conversion (Hesselson et al., 2011). Recently, exciting results presented by Baeyens et al. demonstrate that transient administration of epidermal growth factor (EGF) and ciliary neurotrophic factor (CNTF) stimulates the conversion of terminally differentiated acinar cells to β-like cells in adult mice with chronic hyperglycemia (Baeyens et al., 2014).

Besides acinar cells, other endocrine cells such as α cells have also been shown to be able to reprogram into β cells. Ectopic expression of *Pax4* under the glucagon promoter was sufficient to convert α cells into β cells (Collombat et al., 2009). Furthermore, under conditions of near complete β-cell ablation using the diphtheria toxin system, similar reprogramming processes are observed (Thorel et al., 2010). α cells have high levels of bivalent epigenetic markers, similar to those seen in embryonic stem cells (ESCs). Inhibition of histone methyltransferases leads to partial α-to-β cell-fate conversion

(Bramswig et al., 2013). Most recently, it was discovered that in the juvenile mice, there were δ-to-β cell conversion under conditions of severe β-cell loss (Chera et al., 2014).

A fourth cell type residing within the pancreas that has been extensively studied for its potential to generate functional β cells is the adult pancreatic ductal cell. Upon pancreatic duct ligation, Neurogenin3 (Ngn3), a pancreatic endocrine-determining transcription factor expressed during embryonic development, was reported to be activated in the duct cells of the ligated pancreas (Xu et al., 2008). However, these Ngn3+ cells did not appear to progress further along the endocrine differentiation pathway (Kopp et al., 2011a; Kopp et al., 2011b; Xiao et al., 2013).

Apart from pancreatic cell types, other endoderm-derived cells types have been reported to sustain the capability of differentiating into β-cell as well. The overexpression of *Pdx1* or the PMN factors mentioned above in liver induces expression of insulin genes (Banga et al., 2012; Ferber et al., 2000). Transient expression of PMN factors similarly promotes rapid conversion of intestinal crypt cells into endocrine cells (Chen et al., 2014). In addition, ablation of *Foxo1* in the intestine generates insulin-producing cells both in mouse and human (Bouchi et al., 2014; Talchai et al., 2012).

One final type of cells that is theoretically capable of differentiating into β cells is the pancreatic tissue resident stem /progenitor cells. It has been reported that in the obese and pregnant individuals, there are increase in β-cell mass and β-cell number, but this increase does not seem to be replication related (Butler et al., 2010; Saisho et al., 2013). Furthermore, in the long-duration type 1 diabetic patients, functional β cells are detected (Oram et al., 2014). Potentially, there could be neogenesis or progenitor cell differentiation

events involved in the expansion of human β cells. Putative adult pancreatic stem/progenitor cells that could be clonally expanded and that have multilineage differentiation potentials have already been found in mouse pancreas (Choi et al., 2004; Inada et al., 2008; Rovira et al., 2010; Seaberg et al., 2004; Smukler et al., 2011; Xu et al., 2008; Zulewski et al., 2001). In Chapter 3, I will discuss a novel cell type that we identified in the mature mouse pancreas that has progenitor cell characteristics. These cells are high in Aldefluor activity (A+) and are positive for Sca-1 (Sca-1+). A+Sca-1+ cells are upregulated during the regeneration of acute pancreatitis; can differentiate to both endocrine and exocrine cell fates in dorsal buds transplantation; secrete signaling molecules that are similar to embryonic pancreatic mesenchyme. The unique features of these A+Sca-1+ cells make them a potential candidate for the derivation of β cells and they may secrete paracrine signals to regulate adult pancreatic homeostasis in both normal and pathological conditions.

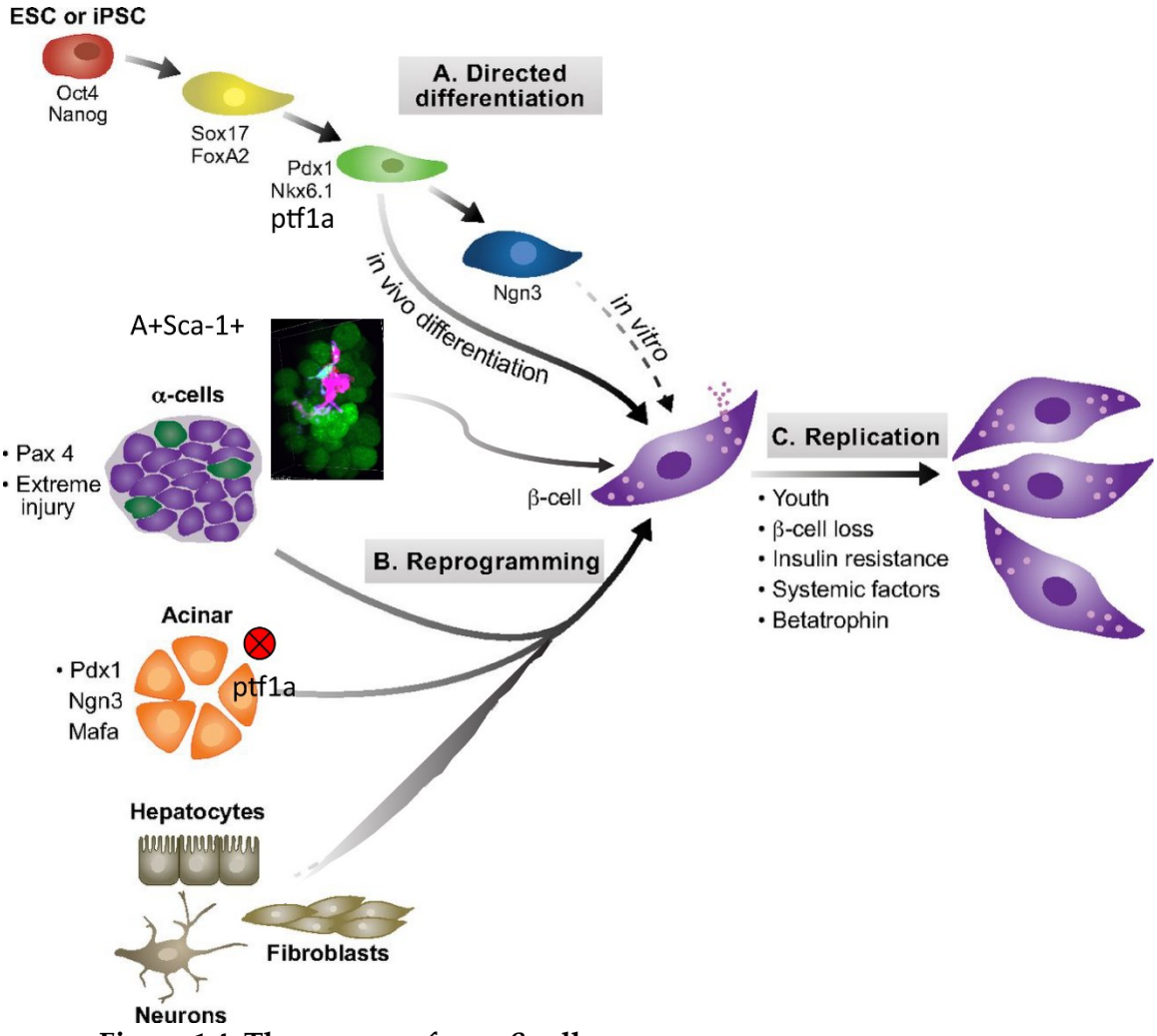


Figure 1.4: The sources of new β cells
(adapted from Pagliuca and Melton (Pagliuca and Melton, 2013).)

Strategies to generate new β-cells. (A) Directed differentiation *in vitro*. Different culture conditions can drive the differentiation of a pluripotent stem cell (red) through the stages of pancreatic differentiation in a manner that mimics normal development. Under current protocols, β cells derived *in vitro* are not functionally mature. A subset of important genes expressed at each stage is listed. Differentiation of A+Sca-1+ progenitor cells into β cells remain to be achieved. (B) Reprogramming of terminally differentiated cell types, such as acinar, *α*-cells, hepatocytes or enterocyte can be used to generate β cells *in vivo*.. (C) Inducing the replication of existing β-cells is the primary strategy for generating new endogenous β cells.

1.5 Pancreatic cancer

Another important disease of the pancreas is pancreatic cancer. Pancreatic cancer is currently the fourth leading cause of cancer death in the United States (American-Cancer-Society, 2014). It is one of the deadliest cancers, with a 5-year survival rate of only 6% (American-Cancer-Society, 2014). Mutations associated with pancreatic cancer are frequently observed in *KRAS*, *CDKN2A*, *TP53*, *BRCA2* and *SMAD4/DPC4* (Bardeesy and DePinho, 2002). Currently, one of the major efforts to understand the mechanisms of pancreatic cancer and provide new therapeutic targets is to identify signature gene mutations and functionally annotate them. In so doing, the knowledge accumulated will serve as a conceptual framework to guide the future analysis of complex aspects of this disease (Biankin et al., 2012).

Up to 85% of pancreatic cancer cases are pancreatic ductal adenocarcinoma (PDAC) (Ghaneh et al., 2002). PDAC primarily exhibits duct-like structures with varying degrees of cellular atypia and differentiation. Pancreatic intraepithelial neoplasia (PanIN) is the most common precursor of PDAC (Hruban et al., 2004). PanINs are classified morphologically into three grades and each grade has its own characteristic molecular signature (Fig. 1.5).

Lineage tracing experiments in mice suggest that PanINs rarely arise from ducts (Brembeck et al., 2003; Kopp et al., 2012), but instead, are most likely derived from acinar cells (De La et al., 2008; Guerra et al., 2007; Habbe et al., 2008; Morris et al., 2010). Acinar cell dysplastic and metaplastic changes are an important initiation step during the evolution of pancreatic cancer (Rooman and Real, 2012).

We are interested in studying the biological function of microRNAs (miRNAs). miRNAs are small noncoding RNAs of 19-25 nucleotides in length (Ambros, 2004; Bartel, 2004; Pasquinelli et al., 2005). They are stable and can be quantified in very small amounts of material (Zen and Zhang, 2012). Different signatures of miRNAs are associated with cancer diagnosis, staging, prognosis and drug response (Calin and Croce, 2006). Furthermore, miRNAs have been shown to function as oncogenes or tumor suppressors and play an active part in tumor initiation and progression (He et al., 2005; Johnson et al., 2005; O'Donnell et al., 2005).

In the pancreas, several miRNA profilings have been carried out to differentiate PDAC, pancreatitis and normal pancreas (Bloomston et al., 2007; Liu et al., 2012; Schultz et al., 2012; Szafranska et al., 2007; Szafranska et al., 2008; Wang et al., 2009). The expression patterns of miR-217 and mir-196a, for example, discriminate normal pancreas, chronic pancreatitis and pancreatic cancer (Szafranska et al., 2007). Moreover, some of the miRNAs have been characterized as potent oncogenes or tumor suppressors in their corresponding pancreatic cancer models (Hwang et al., 2010; Ji et al., 2009; Ryu et al., 2010; Yu et al., 2010; Zhao et al., 2010).

Currently, however, there is no detailed study of the role of miRNAs in normal pancreatic tissue differentiation and cell-fate maintenance. To fill in the gap, we created knockout mice with deletion of the miRNA pathway master regulator, *Dicer*, in the adult pancreatic acinar cells. Combining conditioned *Dicer* deletion with the activation of oncogenic Kras, we further explored the role of miRNAs during pancreatic cancer initiation and progression. *Dicer* knockout induces morphological changes in the acinar cells,

including loss of polarity, epithelial-mesenchymal transition and acinar-ductal metaplasia. *Dicer* dosage is critically regulated during the PanIN initiation. The details will be discussed in Chapter 4. In Chapter 5, we consider the function of one particular miRNA cluster, *mir-216b-217*.

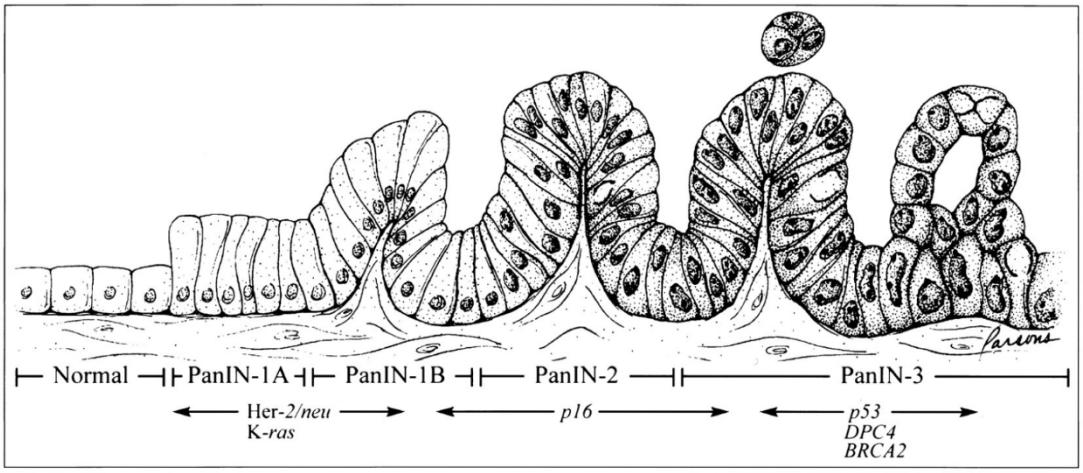


Figure 1.5: The progression of pancreatic cancer
(reprinted from Hruban, et al with permission (Hruban et al., 2000).)
Progression model for pancreatic cancer. Normal duct epithelium progresses to metastatic cancer (left to right) through a series of histologically defined precursors (PanINs). There are stage-specific genetic mutations during pancreatic cancer pathogenesis.

Chapter 2 *ptf1a* lineage tracing in zebrafish.

2.1 Introduction

Zebrafish has emerged as a system to study pancreas organogenesis and has provided new insights on cellular signaling, lineage hierarchy and other developmental mechanisms including β-cell specification during pancreas morphogenesis (Kinkel and Prince, 2009; Tiso et al., 2009; Yee et al., 2005).

Pancreas development in zebrafish shares many features with mammalian pancreas development. The mammalian pancreas develops in two stages. During the first wave of development, referred to as "primary transition", there is early formation of an endocrine population (Herrera et al., 1991; Rall et al., 1973). In mouse, starting around Embryonic Day 13.5 (E13.5), dramatic morphogenetic changes occur. This process is called "secondary transition" and is characterized by rapid cellular proliferation and differentiation. The mature endocrine population starts to appear at this stage (Kemp et al., 1972; Pictet et al., 1972).

Pancreas transcription factor 1a (*Ptf1a*) is one of the earliest genes expressed in the prepanceatic field (Hald et al., 2008). Lineage analysis has shown that mouse *Ptf1a* marks a multilineage progenitor pool that generates both endocrine and exocrine progeny during early pancreatic organogenesis. Between E13.5 to E18.5 the lineage contributions of the Ptf1a progenitor pool become progressively restricted, and by E18.5, *Ptf1a* exclusively labels cells that are destined to become acinar cells (Kawaguchi et al., 2002; Pan et al., 2013). In *Ptf1a* null mice, the growth of pancreas is severely retarded; there is complete lack of acinar cells and the endocrine cells become redistributed to spleen (Burlison et al.,

2008; Krapp et al., 1998). In addition, in the *Ptf1a*-deficient state, progenitor cells that should have been directed to the pancreatic fate instead become incorporated into the duodenum or common bile duct (Burlison et al., 2008; Kawaguchi et al., 2002).

Similar to mammals, the zebrafish pancreas develops from two distinct anlagen arising from foregut endoderm, termed the dorsal and ventral pancreatic buds (Field et al., 2003). While these buds in mammals contain admixed endocrine and exocrine elements, there seems to spatial segregation of early zebrafish endocrine and exocrine pancreatic lineages. The fish dorsal bud develops first and gives rise to a primary endocrine cluster known as the principal islet, while the ventral bud appears later and has traditionally felt to be responsible for generating the exocrine ductal and acinar lineages as well as a later population of endocrine cells (Field et al., 2003; Hesselson et al., 2009; Lin et al., 2004). In zebrafish, the early dorsal bud-derived endocrine population maybe equivalent to the endocrine cells originating from primary transition in the mammalian pancreas; while the vast majority of mature hormone-producing cells are derived from the secondary transition (Hesselson et al., 2009; Parsons et al., 2009). With respect to progenitor cells responsible for generating differentiated pancreatic cell types, two populations have been defined in zebrafish: a Notch-responsive population and a *ptf1a*-expressing population (Wang et al., 2011). Lineage tracing studies have demonstrated that zebrafish Notch-responsive cells (PNCs) differentiate to ducts, centroacinar cells and endocrine cells during the secondary transition (Parsons et al., 2009; Wang et al., 2011), although it remains unclear whether these PNCs may arise from ptf1a-expressing cells. Indeed, the precise contributions of

ptf1a-expressing progenitor cells to different pancreatic lineages has not yet been determined.

Previous work has clearly established that zebrafish *ptf1a* plays an important role in the establishment of acinar cell fate, equivalently to the mammalian counterpart (Lin et al., 2004). Furthermore, the early endocrine population generated during primary transition is independent of *ptf1a* (Lin et al., 2004; Zecchin et al., 2004). But the role of *ptf1a* in endocrine specification during secondary transition remains unclear. Data has shown that a reduced level of *ptf1a* is more favorable for endocrine differentiation (Dong et al., 2008). However, fish lacking *ptf1a* fail to survive to the point of secondary islet formation, leaving open the question of whether these cells are in any way *ptf1a*-dependent. To address these issues and to trace the fate of zebrafish *ptf1a*-expressing cells, we generated an inducible *ptf1a:creER*^{T2} transgenic fish line using BAC recombineering, and employed this line to complete formal lineage tracing studies. Early lineage labeling confirmed that *ptf1a*-expressing progenitors contribute primarily to the acinar cell lineage. However, we also identified contributions to pancreatic Notch-responsive cells (PNCs) and endocrine cells during development. We also demonstrated that *ptf1a* lineage labeled cells gave rise to new β cells during regeneration. Interestingly, heterozygous *ptf1a*$^{sa126/wt}$ mutant fish displayed enhanced contributions of *ptf1a* lineage-labeled cells to the PNC and endocrine cell fates. In addition, we observed that, in the absence functional Ptf1a, *ptf1a* lineage-labeled cells were converted into gall bladder and other non-pancreatic cell types. In summary, we showed that in the zebrafish pancreas, the early *ptf1a*-expressing cells displayed limited multipotency during development and regeneration. Under conditions where *ptf1a* dosage

was reduced, the multipotency of the *ptf1a* lineage was enhanced. These findings confirm conservation of general mechanisms for pancreas development among vertebrates, while highlighting qualitative differences.

Contributions: Dr. Joon Park created the *ptf1a:creER^{T2}* fish line and performed initial characterization. Dr. Joon Park contributed Fig. 2.1. All the rest of the work was carried out by myself.

2.2 Materials and Methods

2.2.1 Generation of transgenic zebrafish lines

We followed the published BAC recombineering method (Suster et al., 2011) to generate the transgene construct *tg*(*ptf1a:creER^{T2}*), abbreviated to *ptf1a:creER^{T2}*, by replacing GFP with *creER^{T2}* elements in the *ptf1a:GFP* construct. The *ptf1a:GFP* construct is derived from genomic BAC CH211-142H2 (Park et al., 2008a; Sharan et al., 2009), which encompasses the zebrafish *ptf1a* coding sequence. *ptf1a:GFP* transgene has been shown to accurately recapitulate the endogenous *ptf1a* expression (Park et al., 2008a; Pashos et al., 2013).

The lineage responder line *tg(ubi:loxP-CFP-loxP-nuc-mCherry),* abbreviated to *ubi:loxP-CFP-loxP-nuc-mCherry* was modified from the previously published line *Tg(T2Kβactin:loxP-stop-loxP-hmgb1-mCherry)^{jh15}* (Wang et al., 2011), where the *βactin* promoter was replaced by the *ubiquitin* promoter (*ubi*) (Mosimann et al., 2011). CFP was also included in the construct to facilitate the identification of the transgenic fish.

Ptf1a$^{sa126/wt}$ fish were obtained from the Sanger Institute Zebrafish Mutation Project (Kettleborough et al., 2013).

Stable *sst:CFP; ins:dsYFP-2TA-nsfB* transgenic fish was established to express CFP under *somatostatin (sst)* promoter, and *nsfB* encoding Nitroreductase was expressed under *insulin (ins)* promoter (Pisharath et al., 2007; Walker et al., 2012).

All fish were maintained under standard conditions. All procedures were performed under the approval of the Johns Hopkins University School of Medicine Animal Care and Use Committee guidelines.

2.2.2 Drug treatment

For the induction of creER activity, 4-Hydroxytamoxifen (4-OHT, T176, Sigma) treatment was performed as previously described (Wang et al., 2011). Briefly, 4-OHT was dissolved in 100% ethanol to create a stock solution of 10 mM. Embryos were placed in a 5 μM solution of 4-OHT in E3 medium from 30 to 54 hours post fertilization (hpf), unless otherwise stated in the text.

For the β-cell ablation experiment, Metronidazole (Met, Sigma, M3761) treatment was performed as previously described (Pisharath et al., 2007). Briefly, Met was dissolved in E3 medium at a concentration of 10mM. Embryos were incubated in the solution for 24 hours from 82 to 106 hpf. Embryo-containing petri dishes were kept in the dark.

2.2.3 Immunofluorescence

For whole mount immunofluorescent staining, larvae were fixed in 4% paraformaldehyde at 4°C overnight. Following fixation the whole gut region was dissected out and blocked for 1 hour with PBS with 0.2% Triton (PBST) and 10% fetal bovine serum. For cryosections, fish were similarly fixed and the gut-intestine system was dissected. Tissues were then immersed in 30% sucrose/PBS, embedded in optimal cutting

temperature (OCT) compound, frozen in liquid nitrogen, and sectioned in 10 μm thickness using a cryostat. Primary antibodies used in this study were: mouse anti-Nkx6.1 (Developmental Studies Hybridoma Bank, F55A12), 1: 100. Guinea pig anti-Insulin (Dako, A0564), 1: 500. Rabbit anti-Glucagon (Dako, A0565), 1:400. Rabbit anti-Somatostatin (Dako, A0566), 1:500. Rabbit anti-DsRed (Clontech, 642496), 1:100. Mouse anti-2F11 (Abcam, ab71286), 1:200. Primary antibodies were incubated at 4°C overnight. After washing with PBST, samples were incubated with secondary antibodies (Jackson Immunoresearch, 1:300) in blocking buffer. Fluorescent images were acquired with Nikon A1 scanning confocal microscope. Cell counting was carried out manually. Briefly, whole mount tissues were scanned by confocal microscope and maximum projections were assembled from Z-stacks. Cell numbers were counted from reconstructed images. Student t-test was implemented for statistical analysis.

2.3 Results

2.3.1 Validation of the *ptf1a:creER^{T2}* driver line

To lineage trace *ptf1a*-expressing cells in a temporarily controlled manner, we engineered a large genomic bacterial artificial chromosome (BAC) spanning the *ptf1a* locus. This BAC has been shown to faithfully recapitulate endogenous *ptf1a* expression (Park et al., 2008a) in pancreas, hindbrain, retina and spinal cord. We replaced *ptf1a* coding sequence with a DNA sequence encoding CreER^{T2}. To facilitate integration of the BAC construct into the genome, a cassette containing the inverted left and right arm of the *Tol2* transposable element was used (Suster et al., 2011). Several independent F1 generation

ptf1a:creER^{T2} transgenic lines were established and were crossed onto the *ubi:loxp-CFP-loxp-nuc-mCherry* reporter line.

In order to screen lines for inducible cre activity, *ptf1a:creER^{T2}*; *ubi:loxp-CFP-loxp-nuc-mCherry* double transgenic larvae were treated with 5 μM 4-Hydroxytamoxifen (4-OHT) at 1 day post fertilization (dpf) for 24 hours and then fixed for imaging at 5 dpf (Fig. 2.1A). These larvae were raised along with untreated controls. At 5 dpf, strong nuc-mCherry signal was observed in 4-OHT-treated larvae, indicating cre-dependent recombination (Fig. 2.1B, d2 and g2). Only rare pancreatic or hindbrain nuc-mCherry signals were detected in larvae without 4-OHT treatment (Fig. 2.1B, d1 and g1).

A single transgenic line with the highest fluorescent intensity upon 4-OHT treatment and lowest incidence of recombination events without 4-OHT treatment ("leakage"), was selected and expanded for further usage.

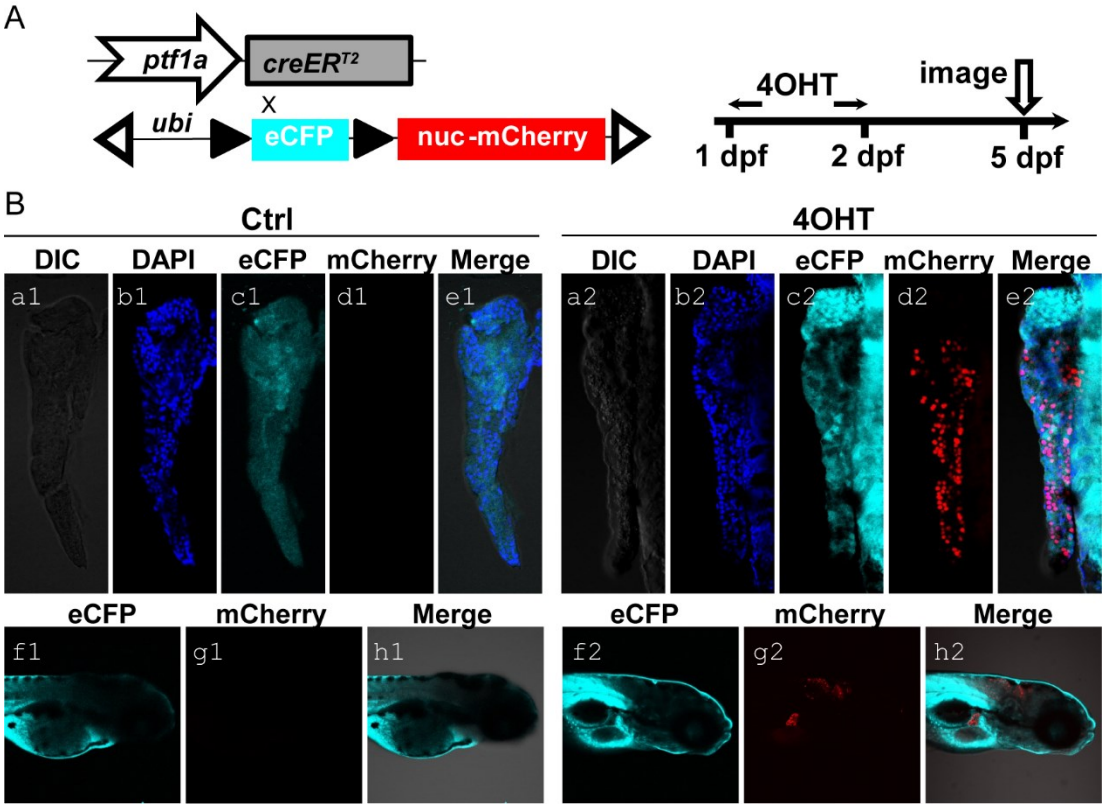


Figure: 2.1. Generation of the ptf1a lineage tracing system and its initial characterization.

(Joon Park)

(A) Schematic of *ptf1a:creERT2* driver and responder *ubi:loxp-CFP-loxp-nuclear-mCherry*. Double transgenic fish larvae were treated with 5 μM 4-OHT in E3 medium at 1 dpf for 24 hours and then fixed for imaging at 5 dpf. These larvae were raised along with untreated controls. (B) At 5 dpf, 4-OHT-treated larvae showed nuclear-mCherry signal (red) in exocrine pancreas (a2-e2) and hindbrain (f2-h2), indicating ptf1a-dependent Cre activity. No pancreatic and hindbrain nuclear-mCherry signals were detected in control larvae (a1-e1, f1-h1). CFP signal (cyan) could be detected ubiquitously (c1, f1, c2, f2). DAPI (blue) stains for nuclei (b1, b2).

2.3.2 The early *ptf1a* lineage has limited contribution to non-acinar cell fates in the zebrafish pancreas

The expression of *ptf1a* is first detectable around 32 hours post fertilization (hpf). It has been reported that ligand-mediated recombination could be detected as early as 2 hours post 4-OHT treatment in the *CreER^{T2}* transgenic zebrafish system (Hans et al., 2009). We treated the lineage-tracing fish with 4-OHT at 30-54 hpf in an effort to label the earliest pool of *ptf1a*-expressing pancreatic progenitor cells (Fig. 2.2A). We subsequently imaged the fish at 6 dpf, when pancreatic architecture is fully formed.

To allow for the correct interpretation of results, we carefully evaluated the tamoxifen-independent recombination (leakage) of the system. We counted the total number of cells undergoing tamoxifen-independent recombination among 32 fish that had not been treated with 4-OHT. In the absence of 4-OHT, an average of 19 (±10) cells per fish were observed to have nuc-mCherry signal. To be noted, this is an estimate of the highest level of leakage, since we only included fish from which we observed leakage events. All of the cells that had recombination events in the absence of 4-OHT treatment displayed the morphology of pancreatic acinar cells. In comparison, among 32 fish that had been treated with 4-OHT, there were on average 377 (± 82) lineage labeled cells per fish. In brief, leakage cells were only a small proportion of the total labeled cells (<5%), and were limited to the acinar lineage. We therefore concluded that our study would not be significantly confounded by tamoxifen-independent recombination, especially as it related to the contribution of *ptf1a*-expressing cells to non-acinar lineages.

We next set out to evaluate the contribution of *ptf1a*–expressing progenitor cells to different pancreatic lineages. We observed broad contributions to the acinar cell lineage, confirming that *ptf1a*–expressing progenitor cells represent the major and perhaps the exclusive source of these cells. We also observed lower magnitude contributions to the PNC and endocrine (principal islet) cell lineages. For the 36 fish we examined, 26 of them, 72% of the total number of fish, did not have any detectable contribution of the ptf1a-expressing lineage to PNCs (Fig. 2.2B; Fig. 2.5F). For the remaining 10 fish, there were on average 8 ptf1a-lineage-labeled cells traced into PNCs (Fig. 2.2C; Fig. 2.5F). This constituted around 5.8% of the total number of PNCs at 6 dpf, which on average were 137 cells per fish (8/137=5.8%). We subsequently surveyed the *ptf1a* lineage contribution to endocrine cells in the principal islet. At 6 dpf, the principal islet contains endocrine cells directly descended from the dorsal bud as well as cells differentiated during the secondary transition (Biemar et al., 2001; Hesselson et al., 2009). For the 21 larvae we assessed, 11 of them, which represented 52% of the total number of fish, did not have any *ptf1a* lineage-labeled cells traced into the principal islet (Fig. 2.2D; Fig. 2.5G). For the 10 remaining larvae where we did observe contributions from *ptf1a-expressing* progenitors to the endocrine lineage, with an average 3.4 lineage labeled cells observable in the principal islet (Fig. 2.2E; Fig. 2.5G). This represents 10% of the endocrine population at 6 dpf, which consists of, on average, 34 cells per principal islet (3.4/34=10%). The large variation of cell counting results in different larvae likely reflected the underlying population heterogeneity (Guryev et al., 2006).

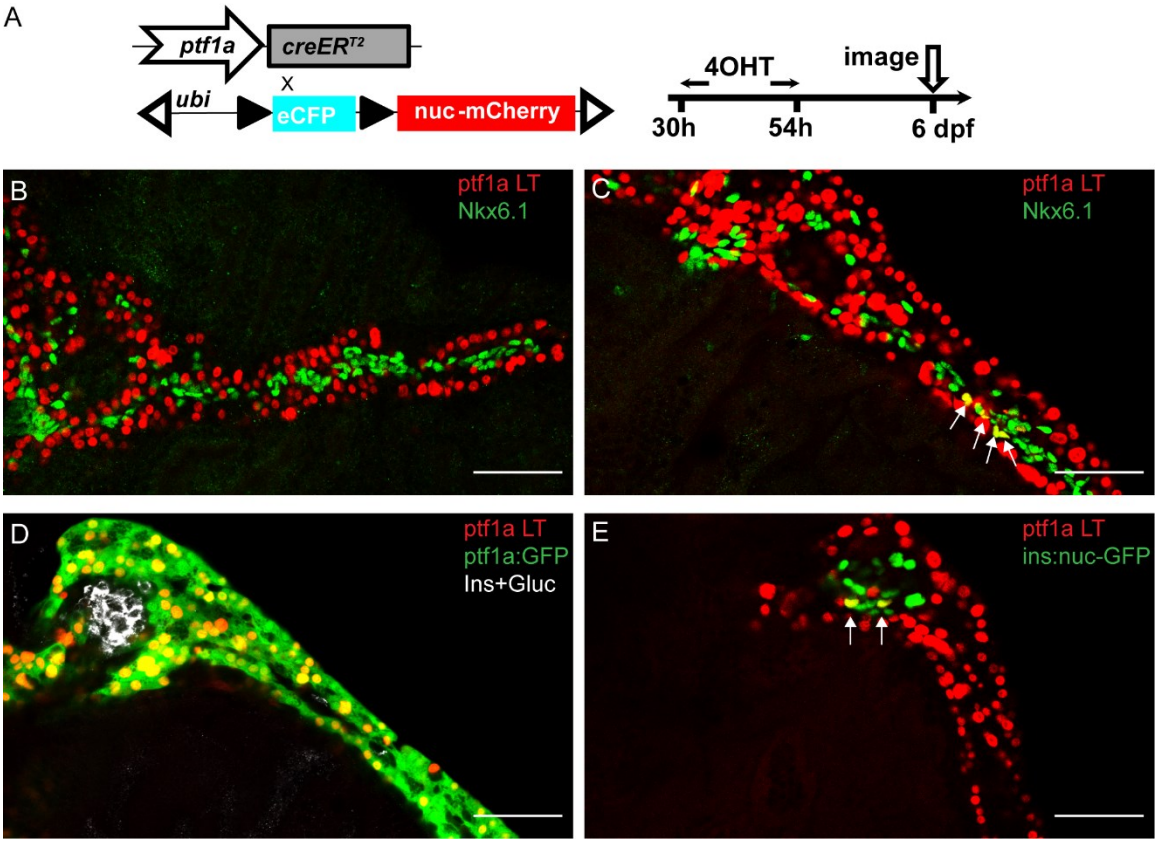


Figure: 2.2. Early *ptf1a* lineage has limited contribution to PNCs and endocrine cells.

(A) Experiment setup. 4-OHT treatment was between 30-54h and larvae were fixed for image analysis at 6dpf. (B-E) In all the panels, *ptf1a* lineage is indicated by nuclear mCherry expression (red). (B) In the majority of pancreata, the ptf1a lineage and PNCs do not have overlaps. Immunofluorescent staining for Nkx6.1 (green) labels PNCs. (C) In some pancreata, *ptf1a* lineage cells are traced into PNCs. Arrows point to colabeling events. Nkx6.1, green. (D) In the majority of pancreata, the ptf1a lineage does not trace into endocrine. *ptf1a:GFP* transgene (green) shows current expression of *ptf1a*, which, at 6 dpf, is limited to acinar cells. Insulin (Ins) and Glucagon (Gluc), white. (E) In a few pancreata, some of the ptf1a lineage cells overlap with signals from the *ins:nuc-GFP* transgene (green), Arrows point to colabeling events. Scale bar, 50 μm.

2.3.3 The *ptf1a* lineage contributes to endocrine cells in juvenile and adult zebrafish

We pulsed 4-OHT from 30-54 hpf, and further traced the *ptf1a* lineage into juvenile and adult fish to assess its contribution during later morphogenesis events. (Fig. 2.3).

In 4-OHT treated fish, at 30 dpf, we saw widespread lineage labeling of acinar cells. In addition, we noted a contribution of *ptf1a*-expressing progenitors to endocrine cells within secondary islets. Interestingly, for some of the secondary islets in which we observed *ptf1a* lineage labelling, almost all endocrine cells within the islets were labeled (Fig. 2.3A, B). We theorized that the *ptf1a* lineage gave rise to the progenitors of those secondary islets, which subsequently had gone through a clonal expansion process to form the secondary islet clusters.

Similarly, in the adult pancreas, we observed isolated as well as clustered *ptf1a* lineage labeled cells located with the islet that were positive for hormone markers (Fig. 2.3C, D). We believe these *ptf1a* labeled endocrine cells emerged during early pancreas development and had since been maintained in the adult pancreas.

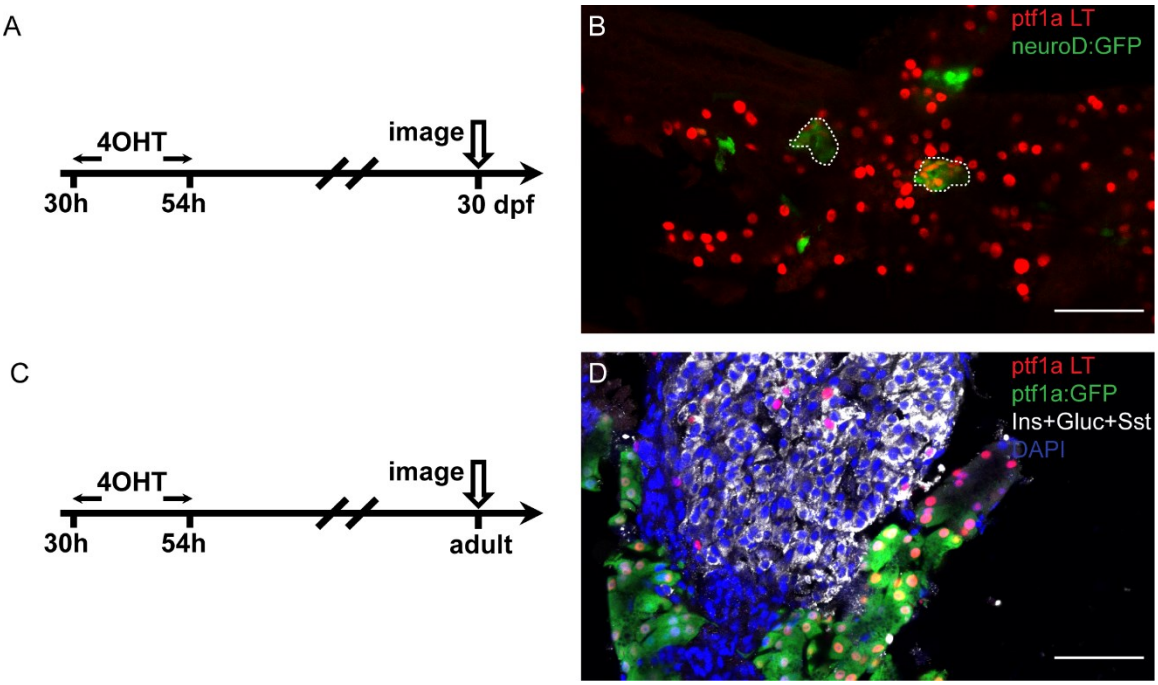


Figure: 2.3. Early *ptf1a* lineage contributes to seconary islet and adult endocrine cells.

(A) Experiment setup for lineage analysis in juvenile fish. (B) A representative section from 30 dpf juvenile lineage-tracing fish. *ptf1a* lineage is labeled by nuclear mCherry expression (red). The secondary islets (outlined) contain a cluster of ptf1a lineage labeled cells. Transgenic marker *neuroD:GFP* (green) labels all endocrine cells. (C) Experiment setup for lineage analysis in adult fish. (D) A representative section from adult lineage-tracing fish. *ptf1a* lineage is labeled by nuclear mCherry expression (red). Some of the endocrine cells have lineage label. Endocrine population is labeled by the mix of antibodies against Insulin (Ins), Glucagon (Gluc) and Somatostatin (Sst), white. *ptf1a:GFP* transgene (green) marks for acinar cells. DAPI, blue. Scale bar, 50 μm.

2.3.4 *ptf1a* lineage contributes to endocrine β-cell regeneration

Having assessed the *ptf1a* lineage during normal pancreas development, we proceeded to determine whether it would contribute to β-cell regeneration as well. To this end, we employed a well-established cell-ablation NTR system to remove β cells. The enzyme nitroreductase (NTR), encoded by the bacterial gene *nfsb*, can convert prodrugs such as metronidazole (Met) to cytotoxins. In our system, we expressed *nfsb* gene under

the control of *insulin* promoter. Subsequently, upon administration of Met, the insulin secreting β cells would be destroyed, with cell death occurring within 6 hours post Met treatment. β-cell regeneration could be observed within 36 hours after Met removal (Pisharath et al., 2007). We crossed *ptf1a:creER^{T2}*; *ubi:loxp-CFP-loxp-nuclear-mCherry* fish with *sst:CFP; ins:dsYFP-2TA-nsfB* fish (Fig. 2.4A).

We treated the resulting triple transgenic fish with 4-OHT from 30-54 hpf and applied Met from 82-106 hpf (Fig. 2.4B). In the control pancreata, YFP-expressing β cells form a compact inner core, intermingled with somatostatin-secreting δ cells and surrounded by other endocrine cell types (Fig. 2.4C). In the Met treated pancreata, much weaker YFP signals were detected within the principal islet (Fig. 2.4D). Some cells contained aggregates with high YFP fluorescence intensity. However, these cells were located out of the boundary of the principal islet and had rounded up, indicating that they were undergoing apoptosis (Fig. 2.4D). We assessed β-cell regeneration at 6 dpf, around 48h after Met removal. At this time point, β cells started to regenerate but there was still a distinct reduction of β-cell numbers in the Met-treated fish (Fig. 2.4E, F). Interestingly, we observed that *ptf1a* lineage contributed to a fraction of the newly generated β cells (Fig. 2.4F). The level of contribution, however, was similar to the control pancreas (Fig. 2.4G), suggesting that the *ptf1a* lineage was not the main source for new β cells.

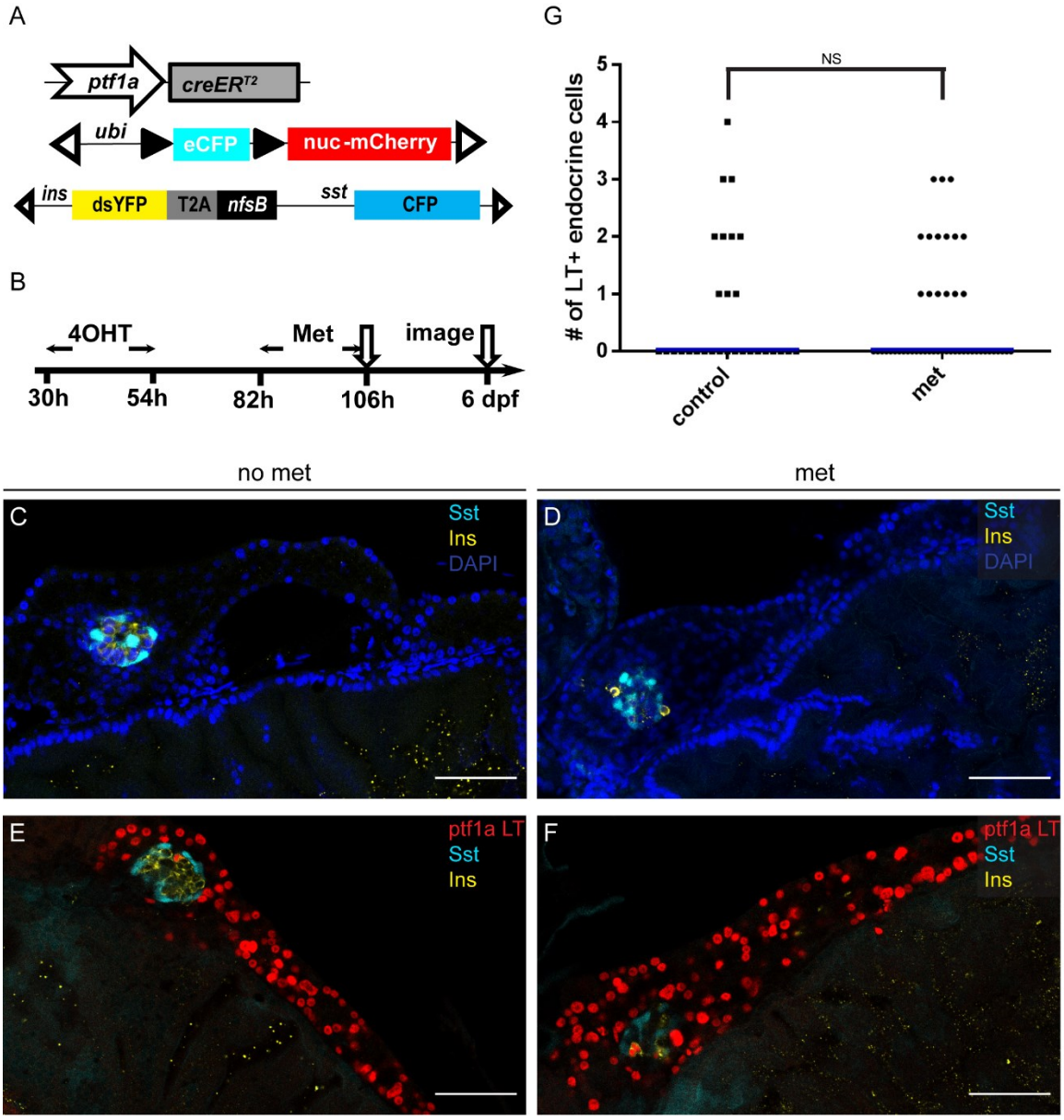

A
ptf1a
creER^T2
ubi
eCFP
nuc-mCherry
ins
dsYFP
T2A
nfsB
sst
CFP
B
4OHT
Met
image
30h
54h
82h
106h
6 dpf
G
NS
# of LT+ endocrine cells
0
1
2
3
4
5
control
met
no met
met
C
Sst
Ins
DAPI
D
Sst
Ins
DAPI
E
ptf1a LT
Sst
Ins
F
ptf1a LT
Sst
Ins

Figure: 2.4. The *ptf1a* lineage contributes to β-cell regeneration.

(A) The triple transgenic line we utilized in the experiment. Besides the *ptf1a* lineage tracing constructs, the fish carries the *nfsB* transgene construct intended for β-cell ablation. Insulin promoter drives expression of destabilized YFP (dsYFP) and *nfsB*. somatostatin (sst) promoter drives the expression of CFP. (B) Experiment setup. (C) In control fish that does not have Metronidazole (Met) treatment, the principal islet has compact architecture. The Insulin-secreting β cells can be find in the inner core of the principal islet interspaced with δ cells. Insulin (Ins), yellow. Somatostatin (Sst), cyan. Nuclei are labeled by DAPI (blue). Notice that the signal from somatostatin expressing cells can be distinguished from the ubiquitin CFP signal due to the higher signal intensity from the former promoter. (D) Immediately after 24h of Met treatment. There is only residues of insulin signal. Some cells have higher than normal Insulin expression. They are apoptotic cells that are generally located on the peripheral of principal islet. Insulin (Ins), yellow. Somatostatin (Sst), cyan. DAPI, blue. (E, F) *ptf1a* lineage is labeled by nuclear mCherry expression (red). 48 h after removal of Met. (E) In untreated fish, there are rare *ptf1a* lineage cells traced into principal islet. Insulin (Ins), yellow. Somatostatin (Sst), cyan. (F) In the Met treated fish, two regenerated β cells have *ptf1a* lineage label. Insulin (Ins), yellow. Somatostatin (Sst), cyan. (G) Quantification of ptf1a lineage labeled cells that are traced into endocrine. There is no significant difference under β-cell regeneration condition.

2.3.5 The *ptf1a* lineage has increased plasticity in the *ptf1a*$^{sa126/wt}$ heterozygous fish

In the mouse, pancreatic cells with different *Ptf1a* gene dosages seem to have altered cell fates (Fukuda et al., 2008; Pan and Wright, 2011). During early mouse pancreas development, it has been shown that intermediate expression of *Ptf1a* marks multipotent progenitor cells, whereas higher *Ptf1a* expression levels are observed in pancreatic progenitors undergoing restriction towards an acinar cell fate (Pan et al., 2013). Similarly, in zebrafish, studies have shown that reduced levels of *ptf1a* seem to promote endocrine differentiation (Dong et al., 2008). Even in the adult fish, reduction of the *ptf1a* activity induces the expression of endocrine-specific genes in the mature acinar cells (Hesselson et al., 2011). To formally characterize the *ptf1a* lineage allocation under conditions of reduced *ptf1a* dosage, we took advantage of the *ptf1a*$^{sa126/wt}$ mutant fish generated by the Sanger Zebrafish Mutation Project (Kettleborough et al., 2013). The *ptf1a*^{sa126} allele contains a nonsense mutation within exon1 of the *ptf1a* coding region (Fig. 2.5A). This mutation is predicted to result in either the expression of a truncated protein or the induction of nonsense-mediated decay (NMD) (Randlett et al., 2013).

We first characterized pancreatic morphology in *ptf1a*$^{sa126/wt}$ heterozygous and *ptf1a*$^{sa126/sa126}$ homozygous fish. Homozygous *ptf1a* mutations phenocopied the previously reported *ptf1a* morpholino phenotype (Fig. 2.5B, C) (Lin et al., 2004). At 5 dpf, *ptf1a*$^{sa126/sa126}$ fish did not have an exocrine pancreas, as assessed by *ptf1a:GFP* transgene; whereas the principal islet appeared normal (Fig. 2.5B, C). In contrast, *ptf1a*$^{sa126/wt}$ heterozygous fish displayed normal pancreatic morphology at 5dpf. These data are

consistent with the prediction that the *pf1a*^{sa126} mutation represents a hypomorphic or functionally null allele.

We next proceeded to perform lineage tracing experiments in *ptf1a*$^{sa126/wt}$ heterozygous fish. Intriguingly, under this condition with reduced *ptf1a* dosage, more *ptf1a* lineage labeled cells were traced into PNCs and the principal islet (Fig. 2.5D-G). For the 29 fish we examined, 10 of them, 10/29=34% of the total number of fish, did not have any *ptf1a*-lineage labelling in PNCs. This percentage is lower than in the wildtype condition, where the corresponding percentage is 72% (Fig. 2.5F). Among the fish where we observed *ptf1a*-lineage labelling in PNCs, on average 17 cells per pancreas, around 13.5% of the total number of PNCs, were labeled by *ptf1a* lineage (Fig. 2.5F). The level of contribution of *ptf1a* lineage to PNCs in the *ptf1a*$^{sa126/wt}$ *ptf1a* heterozygous fish is significantly higher compared with wildtype.

Similarly, we observed that in the *ptf1a*$^{sa126/wt}$ fish, there was a lower percentage of fish in which we did not observe *ptf1a* lineage-labeled cells within the principal islet (22% compared with 52% of fish in the wildtype *ptf1a* condition) (Fig. 2.5G). At the same time, there was a higher percentage of endocrine population labeled by *ptf1a* lineage (18.9% compared with 10% in the pancreata of *ptf1a* wildtype). All of these differences were statistically significant. At the same time, the total number of PNCs and the total area occupied by the endocrine population showed no difference between wildtype and *ptf1a*$^{sa126/wt}$ heterozygous pancreas (Fig. 2.5H, I). Furthermore, there was no correlation between the number of *ptf1a* lineage labeled cells traced into PNCs and the total number of PNCs (data not shown). These data suggest that haploinsufficiency for *ptf1a* promotes

enhanced contribution of *ptf1a*-expressing pancreatic progenitors to non-acinar cell fates, with higher level of regulatory mechanisms acting to regulate the actual number of PNCs and endocrine cells.

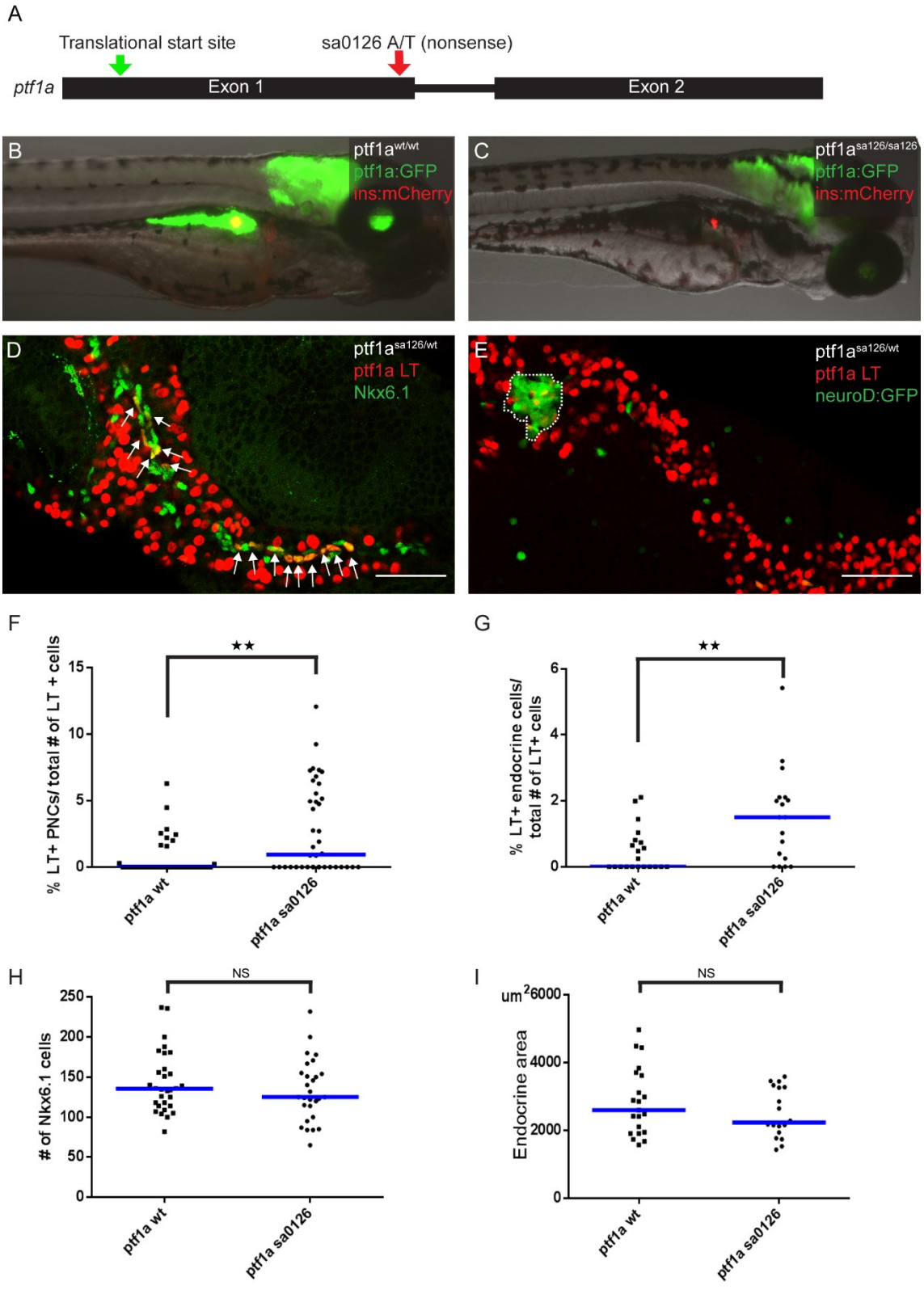

A
Translational start site
sa0126 A/T (nonsense)
ptf1a
Exon 1
Exon 2
B
ptf1a^wt/wt
ptf1a:GFP
ins:mCherry
C
ptf1a^sa126/sa126
ptf1a:GFP
ins:mCherry
D
ptf1a^sa126/wt
ptf1a LT
Nkx6.1
E
ptf1a^sa126/wt
ptf1a LT
neuroD:GFP
F
% LT+ PNCs/ total # of LT + cells
★★
ptf1a wt
ptf1a sa0126
G
% LT+ endocrine cells/ total # of LT+ cells
★★
ptf1a wt
ptf1a sa0126
H
# of Nkx6.1 cells
NS
ptf1a wt
ptf1a sa0126
I
um^2
Endocrine area
NS
ptf1a wt
ptf1a sa0126

Figure: 2.5. The *ptf1a* lineage displays enhanced plasticity in the *ptf1a*$^{sa126/wt}$ fish. (A) The Sanger *ptf1a*^{sa126} allele. This mutant allele has an A to T transition in the first exon of *ptf1a*, introducing a premature stop codon. The *ptf1a*^{sa126} allele either encodes a truncated protein or induce nonsense mediated decay. (B) Wildtype d5 larvae, with *ptf1a:GFP* (green) transgene marks for exocrine pancreas, and *ins:mCherry* (red) transgene marks for β cells within the principal islet. (C) *ptf1a*$^{sa126/sa126}$ homozygous fish do not have exocrine pancreas. Notice the hindbrain and retina signal of *ptf1a:GFP* are preserved. (D, E) ptf1a lineage is labeled by nuclear-mCherry expression (red). (D) In the *ptf1a*$^{sa125/wt}$ fish, more lineage labeled cells can be traced into PNCs. Nkx6.1 (green) is a marker for PNCs. Arrows point to colabling events. (E) In the *pf1a*$^{sa126/wt}$ fish, more lineage labeled cells can be traced into endocrine. *neuroD:GFP* transgene marks for endocrine population. Principal islet is outlined. Notice the mCherry signal within the principal islet. (F) Quantification of *ptf1a* lineage-labeled cells contributing to PNCs. (G) Quantification of *ptf1a*-lineage labeled cells contributing to endocrine. (H) The total number of PNCs and (I) the whole endocrine area are no difference between *pf1a*$^{sa126/wt}$ fish and wildtype fish.

2.3.6 Further cell fate conversion in the *ptf1a*$^{sa126/sa126}$ homozygous fish

In homozygous *ptf1a*$^{sa126/sa126}$ fish, there are no detectable acinar cells and no secondary islets (Fig. 2.5C). Nonetheless, even in the absence of morphologically discernible exocrine pancreas, we still observed a *ptf1a* lineage-labeled population in the region of the intestinal bulb. In order to characterize the identify of these cells, we performed immunolabelling using the 2F11 antibody that recognizes enteroendocrine cells, intrahepatic bile ducts, gall bladder, hepatopancreatic ducts and PNCs (Dong et al., 2007). 2F11 antibody staining indicated that some of the *ptf1a* lineage labeled cells were located within the gall bladder (Fig. 2.6A, B). Moreover, in the homozygous mutant fish, *ptf1a* lineage labeled cells could also be detected in the PNCs as well as the principal islet (Fig. 2.6C, D). Of note, in the *ptf1a*$^{sa126/sa126}$ fish, PNCs were properly specified (Fig. 2.6D). This observation was consistent with the notion that *ptf1a* lineage and PNCs are independently specified (Wang et al., 2011). However, in the homozygous mutant fish, PNCs remained as a cluster of cells adjacent to the principal islets, and the total number of PNCs was significantly reduced (Fig. 2.6D). These data suggest that *ptf1a* lineage-derived acinar cells may provide a supporting cellular framework required for the normal proliferation and migration of PNCs and establishment of the ductal network. The reduced number of PNCs is not likely to reflect a direct requirement for *ptf1a* in a fraction of PNCs, since under wildtype *ptf1a* conditions, there were only around 5% of PNCs were labeled by *ptf1a* lineage. Alternatively, other cell types mediating the effect of acinar cells to PNCs might also be compromised in the *ptf1a*$^{sa126/sa126}$ fish.

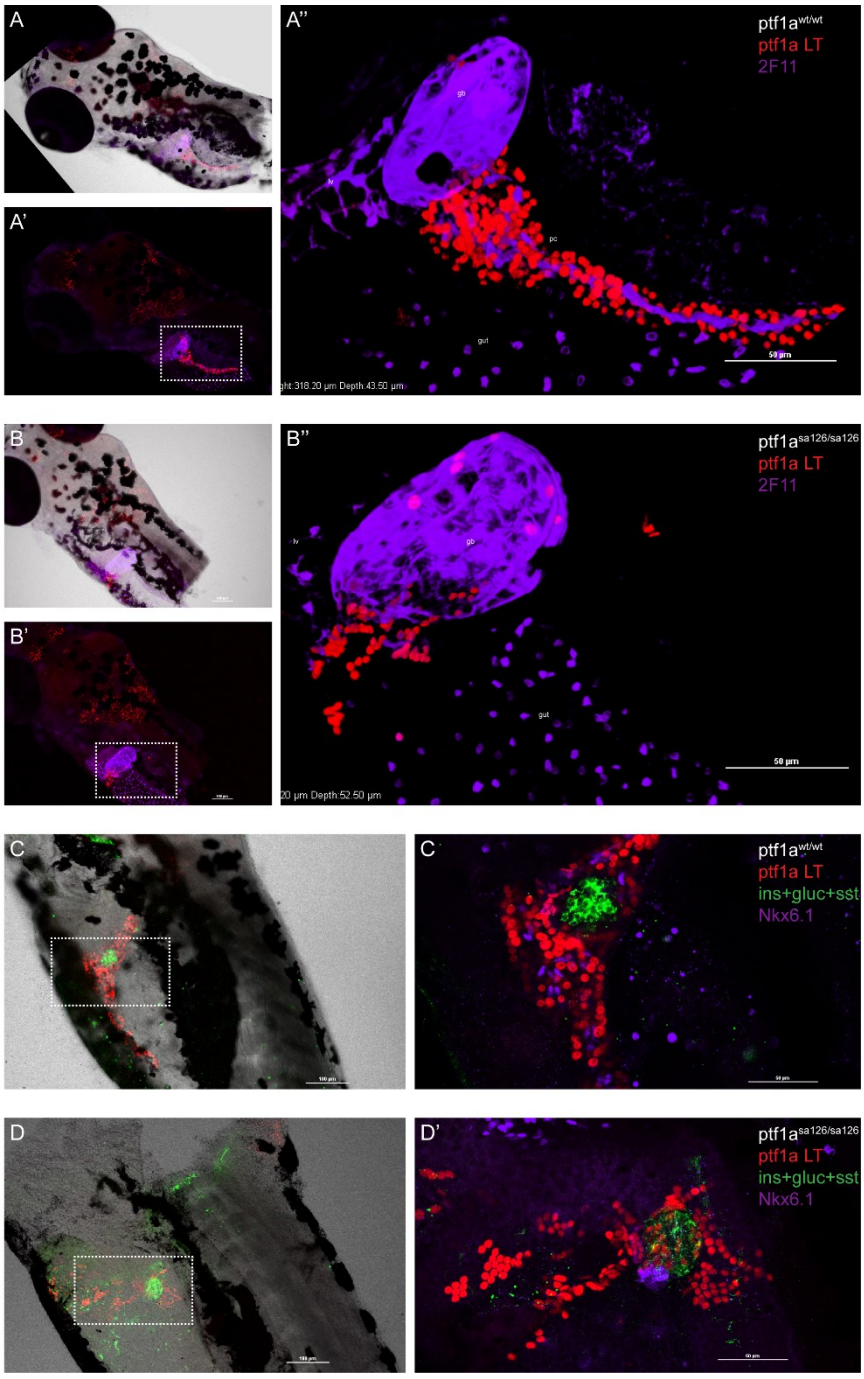

A
A'
A''
ptf1a^wt/wt
ptf1a LT
2F11
gb
pc
gut
50 µm
B
B'
B''
ptf1a^sa126/sa126
ptf1a LT
2F11
gb
gut
50 µm
C
C
ptf1a^wt/wt
ptf1a LT
ins+gluc+sst
Nkx6.1
D
D'
ptf1a^sa126/sa126
ptf1a LT
ins+gluc+sst
Nkx6.1

Figure: 2.6. *ptf1a* lineage analysis in the homozygous *ptf1a*$^{sa126/sa126}$ fish.

In all the panels, *ptf1a* lineage labeled cells are shown in red. **(A)** In wildtype zebrafish, *ptf1a* lineage labelling can be observed in the hindbrain and pancreas (A'). A'' is a zoomed-in, 3D-reconstructed view of the boxed region in A'. 2F11 (purple) labels gall bladder, liver, pancreatic PNCs, and some structure in the gut bulb. Notice that no lineage labeled cells can be observed in the gall bladder. **(B)** In the *ptf1a*$^{sa126/sa126}$ fish, there is no exocrine pancreas structure. Some of the lineage labeled cells can be observed in the wall of gall bladder. In addition, there is another unidentified population right next to the gall bladder. B'' is a zoomed-in, 3D-reconstructed view of the boxed region in B'. 2F11, purple. The gall bladder is also enlarged in the homozygous mutant fish compared with wildtype. Also, in the *ptf1a*$^{sa126/sa126}$ fish, there is still hindbrain lineage labelling. **(C-D)** insulin, glucagon, somatostatin (green) labels endocrine population. Nkx6.1 (purple) labels PNCs. **(C)** In the wildtype zebrafish pancreas, the majority of lineage labeled cells are in the acinar population. PNCs can be observed in the middle of the pancreas. **(D)** In the homozygous *ptf1a* mutant fish, the number of PNCs is reduced and they do not migrate. *ptf1a* lineage labeled cells can be detected in the principal islet and some of the PNCs. gb, gall bladder. lv, liver.

2.4 Discussion

In the present study, we generated a new *ptf1a* fate-mapping fish and carried out formal *ptf1a* lineage analysis in developing, regenerating and ptf1a-deficient pancreas. We observed a major contribution of the *ptf1a* progenitor lineage to later appearing acinar cells, and a minor contributions to PNCs and endocrine cells during development. The *ptf1a* lineage also contributed to β cells during regeneration. Furthermore, different dosages of *ptf1a* influenced the magnitude of non-acinar lineage contributions. In heterozygous *ptf1a*$^{sa126/wt}$ fish, we observed a larger contribution from the *ptf1a* progenitor lineage to the PNC and endocrine compartments. As in the mouse, a complete absence of *ptf1a* leads to incorporation of *ptf1a*-expressing cells into the gall bladder and other foregut tissues.

2.4.1 Clonal expansion of secondary islets

In the secondary islets where we observed *ptf1a* lineage labelling, there was always a cluster of lineage traced cells detected. The low probability of *ptf1a* lineage labelling in the endocrine population made it almost certain that this clustered labelling pattern originated from clonal expansion events. Presumably some of the secondary islets originated from single *ptf1a* positive progenitors. Subsequently, theses progenitor cells undergo clonal expansion. This is consistent with previous studies showing that newly generated secondary islets arise from single cells along the Notch-expression domain, and that these cells are highly proliferative (Parsons et al., 2009).

2.4.2 *ptf1a* lineage and Notch-responsive lineage

We used Nkx6.1 as a surrogate marker for PNCs in the current study. We observed that in the zebrafish pancreas, immunofluorescent labelling for Nkx6.1 completely

overlaps with PNCs marked directly by the transgene *TP1:eGFP* (Huang et al., 2014). In mouse, it has been shown that Ptf1a and Nkx6.1 reciprocally repress the alternative lineage, with the former directing cellular programming towards the acinar fate; whereas the latter promotes an endocrine fate (Schaffer et al., 2010). We believe that the same mechanism is applicable in the zebrafish pancreas. Specifically, we observed that in the *ptf1a*$^{sa126/wt}$ fish where *ptf1a* dosage is partially reduced, there was a significantly greater *ptf1a* lineage contribution to PNCs. PNCs have been shown to be the precursors of endocrine population in zebrafish and inhibition of Notch signaling in PNCs leads to endocrine differentiation (Wang et al., 2011). At present, however, it is unclear whether the increased contribution of the *ptf1a* lineage to the endocrine lineage in the *ptf1a* heterozygous fish went through an intermediated PNC-stage or direct transdifferentiation.

2.4.3 Different levels of *ptf1a* regulate the multi-lineage potential of *ptf1a*-expressing progenitors

With very low levels of *ptf1a* in the *ptf1a*$^{sa126/sa126}$ fish, endocrine cell differentiation still occurred and the principal islet structure was maintained. This result is similar to previously reported *ptf1a* morpholino phenotype (Lin et al., 2004), but different from observations in human and mouse in which low level of *ptf1a* reduces the endocrine cell numbers and alters their distribution (Burlison et al., 2008; Fukuda et al., 2008; Stoffers et al., 1997). This may reflect the different developmental programming of zebrafish pancreas (Tiso et al., 2009). In zebrafish, the early endocrine cells within the principal islet arise from a very early primary transition. At the time when these cells are specified, *ptf1a* has not started to be expressed. The other likely scenario is that in both *ptf1a* morphant and

ptf1a$^{sa126/sa126}$ homozygous fish, there still exists some expression of *ptf1a* to support the proper endocrine lineage differentiation. This possibility is supported by a recent study on the requirement of *ptf1a* expressing cells in formation of retinal neuropil (Randlett et al., 2013). They demonstrated that the combination of *ptf1a*$^{sa126/sa126}$ alleles together with a *ptf1a* translational blocker morpholino are required to reveal the *ptf1a* null phenotype (Randlett et al., 2013).

In the Sanger homozygous fish, the *ptf1a* lineage converted to gall baldder and other non-pancreatic fates. This cell-fate conversion is exactly the same as what was observed in mice (Burlison et al., 2008; Fukuda et al., 2008).

Overall, it is apparent from the current work that different cellular fates require different threshold levels of *ptf1a*. Further studies are needed to characterize the mechanisms that are responsive to different levels of *ptf1a*. Our findings have provided an important understanding on the mechanisms of pancreatic development in zebrafish, which in turn will have important clinical implications, especially in guiding the differentiation of pancreatic β-cells *in vitro*.

Chapter 3. Progenitor cells in adult mouse pancreas

3.1 Introduction

Mesenchymal stem cells (MSCs) have been isolated from a variety of tissues, including bone marrow, peripheral blood, adipose tissue, lung, etc. (Lama et al., 2007; Pittenger et al., 1999; Zuk et al., 2002; Zvaifler et al., 2000). MSCs can readily attach to plastic surfaces and expand rapidly *in vitro* (Beyer Nardi and da Silva Meirelles, 2006). They are capable of differentiating into multiple lineages, including osteoblasts, adipocytes, and chondrocytes (Beyer Nardi and da Silva Meirelles, 2006; Dominici et al., 2006; Pittenger et al., 1999). MSCs have shown great therapeutic potential. They not only directly contribute to the regeneration of tissues of mesoderm, ectoderm and endoderm lineages (Chamberlain et al., 2004; Ortiz et al., 2003; Prabhakaran et al., 2009), but also secrete salutary paracrine signals (Gnecchi et al., 2005), modulate the host immunological response (Aggarwal and Pittenger, 2005), and provide supportive niche (Chen et al., 2002) to benefit transplant recipients.

In the pancreas, there are two other stromal cell types that have been found to have particularly interesting properties: pancreatic stellate cells (PSCs) (Apte et al., 1998;

Bachem et al., 1998) and pericytes (Crisan et al., 2008). PSCs are the major source of fibrosis in chronic pancreatitis and pancreatic cancer. They have evolved to be a promising target for pancreatic cancer therapy. PSCs contain vitamin A droplets during quiescence. When activated, they lose their vitamin A droplets, start to express α-smooth muscle actin (α-SMA) and other extracellular matrix (ECM) proteins. PSCs express specific markers such as desmin, vimentin, nestin, and glial fibrillary acidic protein (GFAP). (Apte et al., 2004; Casini et al., 2000; Haber et al., 1999; Masamune et al., 2009; Omary et al., 2007). Pericytes, on the other hand, have long cytoplasmic processes, wrap around endothelial cells and express molecular markers such as desmin, α-SMA, platelet-derived growth factor-B (PDGF-B), and NG2, a proteoglycan associated with pericytes during vascular morphogenesis (Hellstrom et al., 1999; Nehls and Drenckhahn, 1991; Ozerdem et al., 2001). Similar to MSCs, pericytes have been shown to have multilineage differentiation potential (Crisan et al., 2008). Furthermore, pericytes exhibit abnormalities in pancreatic cancer (Morikawa et al., 2002).

The initial plan of the current study was to identify and thoroughly characterize adult pancreatic progenitor cells. Adult pancreatic progenitor cells, if they exist, would promise a giant leap in the generation of new β cells for cellular replacement therapy for the treatment of diabetic patients (Bouwens et al., 2013). Putative progenitor cell populations have been reported among acinar cells, ductal cells, centroacinar cells, as well as endocrine cells (Choi et al., 2004; Inada et al., 2008; Minami et al., 2005; Rovira et al., 2010; Seaberg et al., 2004; Smukler et al., 2011; Xu et al., 2008; Zulewski et al., 2001). However, these reports have largely been based on *in vitro* culture and they suffer from

incomplete lineage characterization *in vivo*. Hence we do not know for sure where these progenitor cells originate from and what their endogenous functions are. The jury is still out concerning whether there exists in adult pancreatic tissue any resident progenitor cells. To explore new populations of progenitor cells in pancreas, we evaluated markers that have been reported to be enriched in stem /progenitor cells within pancreas and other tissue – both in the normal as well as neoplastic tissues undergoing cancer initiation and progression. These markers include: Aldefluor activity (Ginestier et al., 2007; Rasheed et al., 2010; Rovira et al., 2010), CD24 (Al-Hajj et al., 2003; Li et al., 2007), CD44 (Al-Hajj et al., 2003; Li et al., 2007; Prince et al., 2007), CD49f (Sugiyama et al., 2007), CD133 (Prominin-1) (O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2004; Sugiyama et al., 2007), c-kit (Ellison et al., 2013; Esposito et al., 2002), c-Met (Li et al., 2011), EpCam (Gires et al., 2009; Li et al., 2007; Munz et al., 2009), and Sca-1 (Holmes and Stanford, 2007). Corresponding populations of cells from adult mouse pancreata were isolated and cultured in sphere-forming condition (Rovira et al., 2010; Visvader and Lindeman, 2008; Wang et al., 2013). In doing so, we identified a unique type of stromal cell residing in the adult mouse pancreas. These cells were characterized by high Aldefluor activity (A+) and Sca-1 positivity (Sca-1+). They had long cytoplasmic processes and were found adjacent to blood vessels. They shared some features with mesenchymal stem cells, such as their sphere-forming capability, plastic affinity and multi-lineage differentiation potential. However, they did not have all the canonical MSC surface markers, such as CD105 and CD90. Similarly, A+Sca-1+ cells have some characteristics of stellate cells and pericytes, but they did not completely fit into the profiles of either category of cells. A+Sca-

1+ cells were upregulated during cerulein-induced acute pancreatitis. Cytokine arrays revealed that A+Sca-1+ cells secreted factors similar to embryonic pancreatic mesenchyme. We, therefore, propose that A+Sca-1+ cells function as a niche to maintain normal pancreas homeostasis. Their mesenchymal differentiation potential *in vitro* and their upregulation during pancreatitis imply that they may also play a role in fibrosis during pathological change of pancreas.

Contributions: Dr. Meritxell Rovira made the initial observation that Aldefluor positive cells had increased sphere-forming capability. I subsequently repeated this result and further advanced the analyses as noted below. The following chapter represents my own work.

3.2 Materials and Methods

Sections 3.1.1 to 3.1.2.6 are adapted from Springer Methods in Molecular Biology, 980, 2013, pp 281-290, Sphere-forming assays for assessment of benign and malignant pancreatic stem cells, Yue J. Wang, Jennifer M. Bailey, Meritxell Rovira, Steven D. Leach, figure number: 1; with kind permission from Springer Science and Business Media (Wang et al., 2013).

3.2.1 Materials

1. Isofluorane.

2. Hank's balanced salt solution (HBSS) w/o Calcium and Magnesium (Gibco 14175-095).

3. Phosphate Buffered Saline (PBS).

4. Collagenase P,Roche 11213865001.

5. Trypsin.

7. Trypan blue.

8. Aldefluor kit, STEMCELL Technologies 01700

9. Pancreatic stem cell culture medium

Basal medium is DMEM/F12 (1:1) Gibco 11039. This medium is supplemented with:

FBS, Gibco

NEAA, Gibco 11140

N2, Stemcell Technologies 07152

B27, Invitrogen 17504-044

LIF, Stemcell Technologies 02740a

EGF, Peprotech AF-100-15

FGF2. Invitrogen 13256-029

Equipment

1. Surgical tools.

2. Polypropylene mesh (100 μm and 500 μm).

3. 37℃ waterbath.

4. Inverted microscope.

5 Haemocytometer.

6. Centrifuge at 4℃.

3.2.2 Methods

Carry out all procedures when possible in a sterile hood to minimize chances of contamination of cultures.

3.2.2.1 Media preparation for culturing adult mouse pancreas

1. 50ml HBSS with 5%FBS. Store on ice.

2. 10ml HBSS. Store on ice.

3. 10ml PBS with 5%FBS. Store on ice.

4. Pre-warm 5ml Trypsin/EDTA 0.05% in 37℃ in water bath.

5. 10ml Collagenase P, 1-1.5 mg/ml in HBSS.

6. Pancreatic stem cell culture medium

Component	Volume (μl)	Final concentration
DMEM/F12		Add up to 50ml
FBS	1500	3%
Non Essential Amino Acid	500	1×
Pen/Strep	500	100 U/ml
N2 Supplement	500	1×
B27 Supplement	1000	1×
EGF		20ng/ml
FGF_2		20ng/ml
LIF		10ng/ml
Beta-mercaptoethanol		100 μM

*The activity of the growth factors decreases with time. It is preferred to prepare medium fresh each time. Do not keep media longer than 7 days when stored at 4℃.

3.2.2.2 Single cell isolation from adult mouse pancreas.

1. Anesthetize the 6-12 week old adult mouse with isofluorane.

2. Dissect the pancreas and transfer it to a petri dish with HBSS on ice. Wash off any blood.

3. Transfer pancreas to an empty petri dish and inject 1 ml of Collagenase P at various points with 1 ml insulin syringe and 28 gauge needle.

4. Mince the pancreas into 1-5mm pieces using scissors and transfer everything into a 50 ml falcon tube with 4 ml of Collagenase P (1-1.5 mg/ml).

5. Place the 50ml tube in a 37℃ water bath. Shake gently every 3 min for 11 min total. The digestion progression can be monitored by sampling and observation under an inverted microscope. Digestion should stop when no big tissue chunks remain.

6. Stop the reaction by placing the tube on ice and adding 5ml of HBSS with 5% FBS.

7. Centrifuge at 720 x g for 2min at 4℃. Decant the supernatant and resuspend the pellet with 5ml of cold HBSS with 5% FBS. Centrifuge at 720 x g for 2min at 4℃.

8. Decant the supernatant and resuspend the pellet with 5ml cold HBSS. Filter through a 500 μm mesh. Wash the tube with 5ml cold HBSS and filter into the same tube. Centrifuge at 720g for 2 min at 4℃.

9. Decant the supernatant and trypsinize with 0.05% Trypsin/EDTA for no longer than 5min in a 37℃ water bath. This is the most critical step.

10. Stop the reaction by placing the tube on ice and adding 5ml of cold PBS with 5% FBS.

11. Centrifuge at 720 x g for 2min at 4℃. Decant the supernatant and resuspend the pellet with 5ml of cold PBS with 5% FBS. Centrifuge at 600 x g for 3 min at 4℃.

12. Decant the supernatant and resuspend the pellet with 5ml cold PBS with 5% FBS. Filter through a 100 μm mesh. Wash the tube with 5ml cold PBS with 5% FBS and filter into the same tube. To analyze cell viability and to calculate the number of cells, take 10 μl sample into 90 μl of Trypan blue. Centrifuge the rest at 600 x g for 3 min at 4℃.

3.2.2.3 Labeling of pancreatic cells with Aldefluor reagent.

1. Calculate the viable cell concentration with a hemacytometer. Viable cells are determined by Trypan Blue dye exclusion.

2. Re-suspend cells with Aldefluor buffer at 1 million cells per ml.

3. Take out 500 μl cells as nonstaining control.

4. Prepare a 15 ml conical tube labeled with DEAB. Add 5 μl of DEAB into this tube. Fasten the cap to prevent evaporation.

5. Add 5 μl of Aldefluor substrate per each ml of cells.

6. Immediately transfer 500 μl of cells from step 5 into the tube labeled with DEAB.

7. Put the nonstaining tube, the Aldefluor tube and DEAB tube in a 37℃ cell culture incubator and incubate for 40-50 min.

8. To stop the reaction, put the sample on ice.

9. Centrifuge at 600 x g for 3min at 4℃.

10. Decant the supernatant. Re-suspend the cell pellets with PBS, 5%FBS and filter with a 40 micron cell strainer.

3.2.2.4 Flow cytometry

Set up proper gates according to the instructions in Aldefluor kit.

Sort onto a plate or into 5ml tubes. Collect A+ and A- populations.

3.2.2.5 Pancreatosphere culture

Seed the cells (6000 cells/ml) on ultra-low attachment plates. Incubate at 37°C with 95% air and 5% CO2. Culture cells for 5-7 days.

3.2.2.6 Preparation of whole-mount pancreatosphere for immunofluorescent labeling

1. Transfer the culture medium with pancreatospheres to 15ml conical tube. Allow the spheres to settle by gravity.

2. Remove the supernatant without disturbing the bottom and mix gently with 4% PFA. Fix the spheres for 20min at room temperature.

3. Remove the supernatant and rinse with 10ml of PBS. Allow the spheres to settle.

4. Remove the supernatant and add 5ml of PBS with 0.2%Triton and 10%FBS. Block for 1h at room temperature.

5. Remove the supernatant and add primary antibody. Incubate at 4°C overnight.

6. Directly add 10ml of PBST with 10%FBS into the sphere tube the next day.

7. Allow spheres to settle. Take out the supernatant and wash the spheres once with PBST with 10% FBS.

8. Remove the supernatant and add seconary antibody. Incubate at room temperature for 2 hrs.

9. Directly add 10ml of PBS into the sphere tube.

10. Allow the spheres to settle. Take out the supernatant and wash the spheres once with PBS.

11. Take out as much of the supernatant as possible. Add DAPI into the sphere tube.

12. Take out the medium with spheres and add it onto a slide circled with PAP pen. Wait until the spheres settle onto slides. Under the microscope, try to find the spheres and remove the surrounding medium.

13. Find spheres under a confocal microscope using the DAPI channel.

3.2.2.7 In vitro differentiation of mesenchymal stem cells

The mutilineage differentiation of A+Sca-1+ cells was carried out following the protocol available from R&D systems, Catolog #SC010.

3.2.2.8 Cerulein induced acute pancreatitis

Acute pancreatitis was induced by administration of 6 hourly intraperitoneal injections of cerulein 50 μg/kg over 2 consecutive days. At indicated time points, mice were sacrificed and acinar cells were prepared for live labeling. All procedures were performed under the approval of the Johns Hopkins University School of Medicine Animal Care and Use Committee guidelines.

3.2.2.9 E12.5 pancreatic dorsal buds culture and microinjection

E12.5 dorsal pancreatic buds were isolated as previously described (Esni et al., 2005). Immediately after dissection, the dorsal buds were injected with Aldefluor-positive or -negative cells freshly sorted from adult mTmG transgenic mouse pancreas. They were subsequently cultured on top of MilliCell-CM insert, in RPMI medium + 10% FBS, with Penicillin and Streptomycin for 7 days. Standard fixation, OCT embedding and immunofluorescence were pursued.

3.2.2.10 Cytokine array

RayBio mouse cytokine antibody array 3 (G-Series) was carried out per the manufacturer's protocol. The G3 array has 62 targets in total:

Axl	BLC (CXCL13)	CD30 Ligand (TNFSF8)	CD30 (TNFRSF8)	CD40 (TNFRSF5)
CRG-2	CTACK (CCL27)	CXCL16	Eotaxin-1 (CCL11)	Eotaxin-2 (MPIF-2/CCL24)
Fas Ligand (TNFSF6)	Fractalkine (CX3CL1)	GCSF	GM-CSF	IFN-gamma
IGFBP-3	IGFBP-5	IGFBP-6	IL-1 beta (IL-1 F2)	IL-10
IL-12 p40/p70	IL-12 p70	IL-13	IL-17A	IL-1 alpha (IL-1 F1)
IL-2	IL-3	IL-3 R beta	IL-4	IL-5
IL-6	IL-9	KC (CXCL1)	Leptin	Leptin R
LIX	L-Selectin (CD62L)	Lymphotactin (XCL1)	MCP-1 (CCL2)	MCP-5
M-CSF	MIG (CXCL9)	MIP-1 alpha (CCL3)	MIP-1 gamma	MIP-2
MIP-3 beta (CCL19)	MIP-3 alpha (CCL20)	Platelet Factor 4 (CXCL4)	P-Selectin	RANTES (CCL5)

SCF	SDF-1 alpha (CXCL12 alpha)	TNF RI (TNFRSF1A)	TNF RII (TNFRSF1B)	TARC (CCL17)
I-309 (TCA-3/CCL1)	TECK (CCL25)	TIMP-1	TNF alpha	Thrombopoietin (TPO)
VCAM-1 (CD106)	VEGF-A			

3.3 Results

3.3.1 Aldefluor (+) Sca-1(+) population in adult mouse pancreas

In a search for potential adult pancreatic progenitor cells, we first evaluated a number of stem cell markers that have been reported in the literature for a variety of tissues. These included: Aldefluor activity, CD24, CD44, CD49f, CD133, c-kit, c-met, EpCam, PDGFR-B, and Sca-1. Interestingly, all of the markers under assessment were found to be expressed by some cells inside the pancreas either by flow cytometry and/or by immunofluorescence.

Sphere-forming assays are widely used in stem cell biology to assess the self-renewal and differentiation potential of a particular cell type (Pastrana et al., 2011). We adapted a protocol similar to that reported for the generation of neurospheres to generate pancreatospheres (Reynolds and Weiss, 1992; Rovira et al., 2010; Wang et al., 2013). Corresponding populations positive for single marker and combinations of markers were isolated by flow cytometry and cultured at clonal density (6,000~10,000 cells/ml of media) in suspension in the sphere-forming media. Among all the markers we tested, the combination of Aldefluor positive (A+) and Sca-1 positive (Sca-1+) highly enriched a sphere-forming population (Fig. 3.1). The Aldefluor reagent offers an approach to isolate

stem/progenitor cells based on their high expression of the enzyme aldehyde dehydrogenase (ALDH) (Corti et al., 2006; Ginestier et al., 2007; Storms et al., 1999). Sca-1 (Ly-6 A/E) is a glycosyl phosphatidylinositol (GPI)-linked cell surface protein found on stem/progenitor cells in multiple tissues and organs including the hematopoietic system (Spangrude et al., 1988), mammary gland (Welm et al., 2002), myocardium (Oh et al., 2003), liver (Petersen et al., 2003) and lung (McQualter et al., 2009).

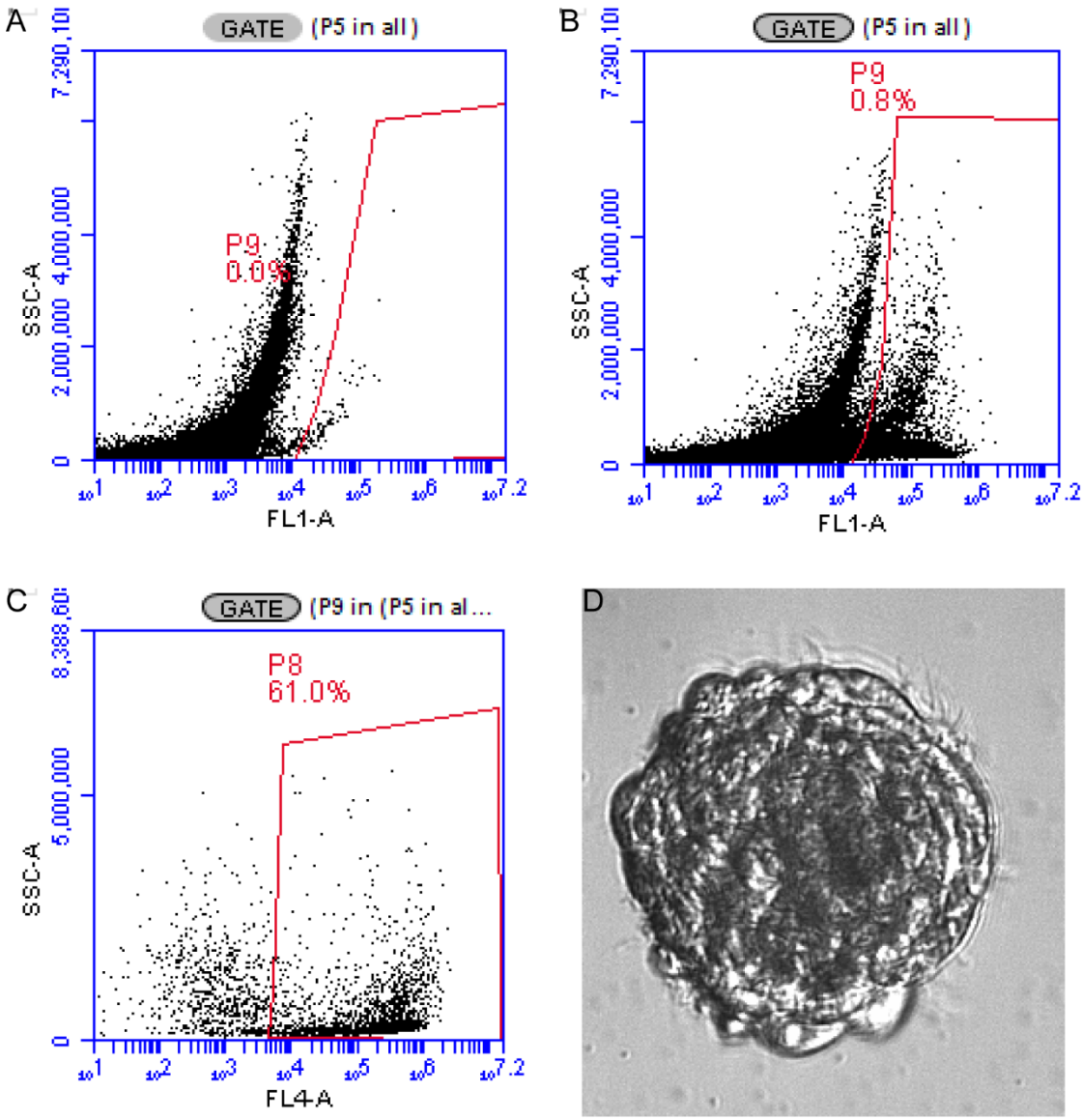


Figure 3.1: Aldefluor (+) Sca-1(+) population in the adult pancreas.
(A) Gating of Aldefluor positive (A+) cells based on Aldefluor + DEAB negative control. (B) Aldefluor labels around 1% of total cells in the adult mouse pancreas acinar preparation. (C) Plot shows only the A+ cells. Among the A+ population, around 50% of them are Sca-1+. (D) Flow sorted A+Sca-1+ cells form pancreatospheres in suspension at day 5.

3.3.2 Aldefluor (+) Sca-1(+) cells have unique features

I performed live labelling and imaging of A+Sca-1+ cells. Light Collagenase P digestion would release the acini from the pancreatic parenchyma but maintain their organization. Subsequent Aldefluor reaction and whole mount cell-surface antibody labelling could be carried out on these intact acini. This procedure preserves the endogenous architecture formed by different pancreatic cell types. Confocal analysis showed that A+Sca-1+ cells had long cytoplasmic processes and were in close proximity to blood vessels (Fig. 3.2A). Apart from being Aldefluor and Sca-1 positive, this population of cells did not display positive staining of the endothelial cell marker (CD31) (Fig. 3.2A, A'), the epithelial cell maker (EpCam) (Fig. 3.2B, B'), the ductal cell marker (*Dolichos biflorus* agglutinin, DBA) (Fig. 3.2C, C') or the pericyte marker (NG2) (Fig. 3.2D). RNAs were extracted from isolated A+Sca-1+ cells. qRT-PCR showed that A+Sca-1+ cells had high expression of vimetin and GFAP, which are known markers of stellate cells (Fig. 3.2E). However, stellate cells are also high in nestin (Omary et al., 2007). On the contrary, A+Sca-1+ cells were low in nestin compared with the unsorted cell population (Fig. 3.2E).

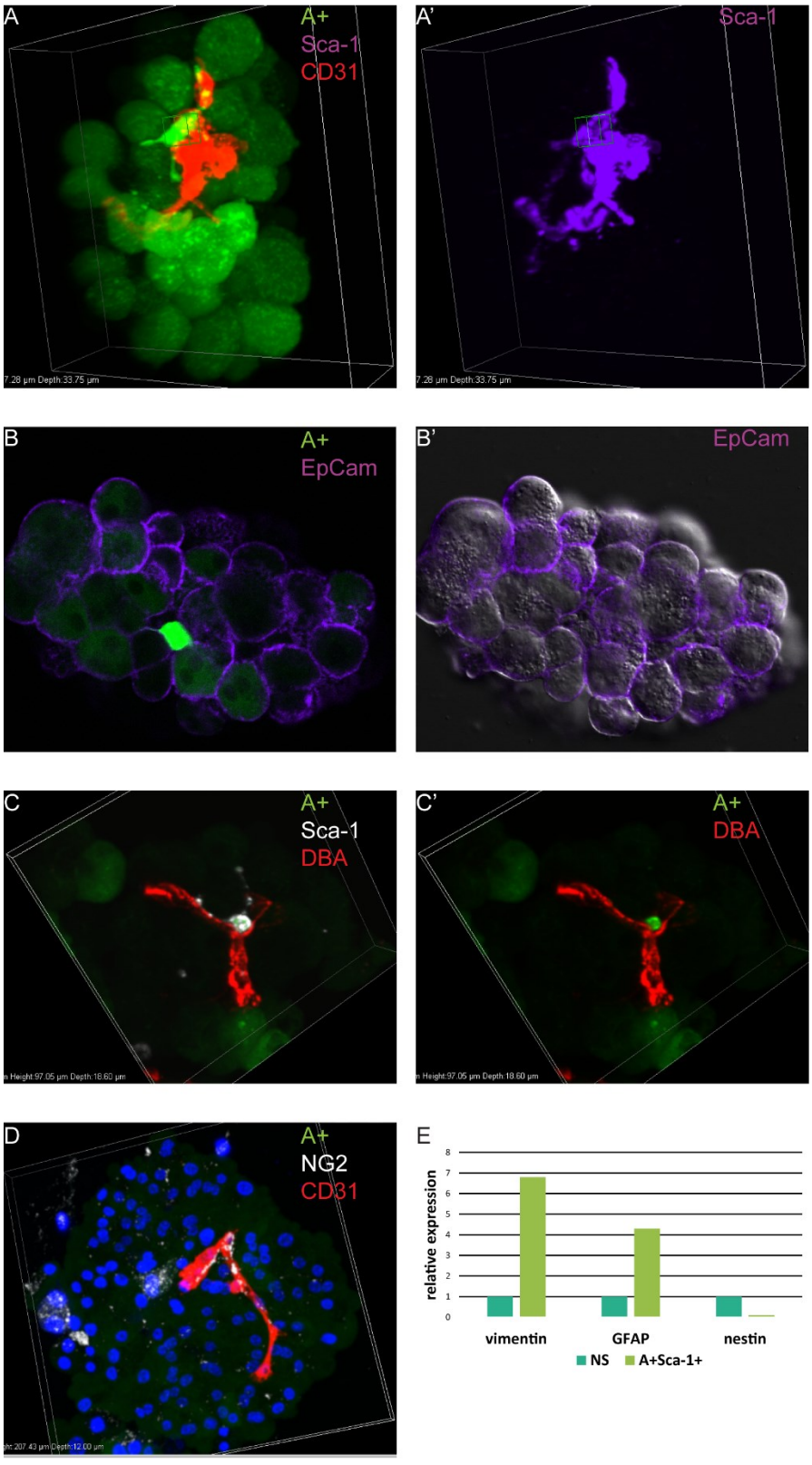

A
A+
Sca-1
CD31
A'
Sca-1
B
A+
EpCam
B'
EpCam
C
A+
Sca-1
DBA
C'
A+
DBA
D
A+
NG2
CD31
E
relative expression
0
1
2
3
4
5
6
7
8
vimentin
GFAP
nestin
NS
A+Sca-1+

Figure 3.2: The Aldefluor (+) Sca-1 (+) population has unique cellular features. (A, A′) A+Sca-1+ cells have long cytoplasmic processes and locate in proximity to blood vessel. Pictures shown are from live-labeling of collagenase digested acini. Aldefluor labeling is shown in green. Notice that there is background labeling in the acinar cells. A+ cells are defined as those cells with high Aldefluor signal, shown in bright green in all the panels. CD31 (red) labels endothelial cells. (B, B′) A+ cells are not epithelial cells. EpCam, purple, labels epithelial cells. EpCam staining is absent in A+ cells. (C, C′) A+Sca-1+ cells are not ductal cells. DBA (red) labels pancreatic duct. Sca-1, white. (D) A+ cells are not pericytes. Pericytes are labeled by NG2 (white). They wrap around CD31+ (red) blood vessels and have distinct morphology compared with A+Sca-1+ cells. (E) qRT-PCR using mRNA isolated A+Sca-1+ cells show that this population has high expression of vimentin and GFAP, but lower expression of nestin compared with the bulk nonsorted population in the adult mouse pancreas.

3.3.3 Aldefluor (+) Sca-1 (+) cells have mesenchymal stem cells properties

A+Sca-1+ cells rapidly proliferated *in vitro*. As previously described, isolated A+Sca-1+ cells formed spheres in suspension within 5 days under sphere-forming conditions at clonal density (Fig. 3.1D, 3.3A). These spheres could be dissociated and passaged. Reseeded single cells from the primary spheres maintained the competence of forming secondary spheres (Fig. 3.3B). Furthermore, A+Sca-1+ cells adhered to plastic and could be passaged for multiple generations as attachment cultures. Under both suspension and plastic attachment culture conditions, A+Sca-1+ cells continued to express Vimentin and GFAP, but also turned on expression of Nestin and SMA (Fig. 3.3, C-E).

Mesenchymal stem cells have been shown to be capable of adipogenic, osteogenic, and chondrogenic differentiation (Dominici et al., 2006). The multilineage differentiation potential of A+Sca-1+ cells were assessed using a Mouse Mesenchymal Stem Cell Functional Identification Kit (R&D Systems), according to the manufacturer's protocol. Strikingly, A+Sca-1+ cells also had the ability to differentiate into adipocytes, osteocytes and chondrocytes, establishing themselves as *bona fide* mesenchymal stem cells (Fig. 3.3, F-H).

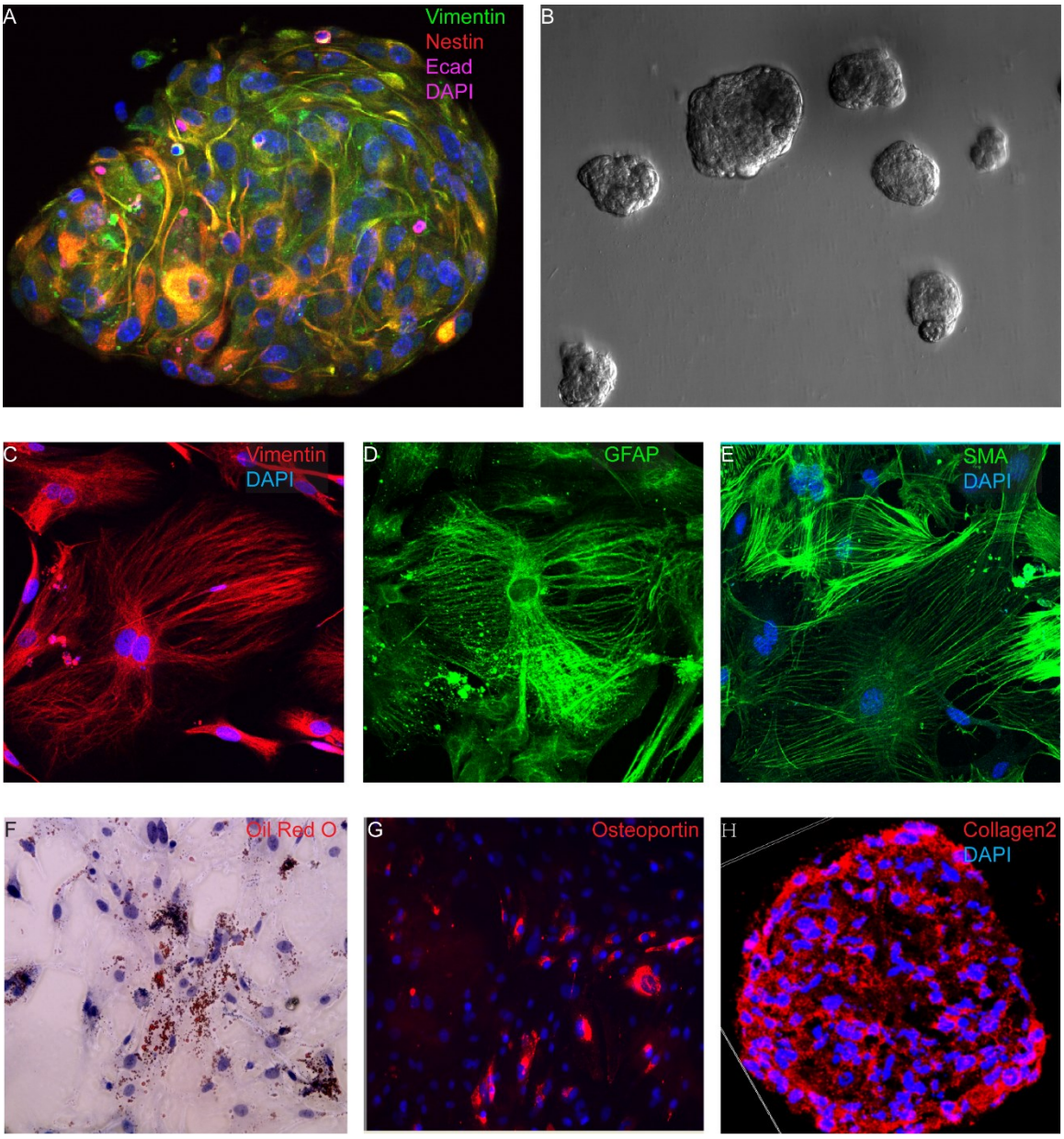

A
Vimentin
Nestin
Ecad
DAPI
B
C
Vimentin
DAPI
D
GFAP
E
SMA
DAPI
F
Oil Red O
G
Osteoportin
H
Collagen2
DAPI

Figure 3.3: Aldefluor (+) Sca-1(+) cells have mesenchymal stem cell properties. (A) Isolated A+Sca-1+ cells form mesenchymal spheres in clonal density. Cells in the sphere express mesenchymal markers Vimentin (green) and Nestin (red), but are mostly Ecad (pink) negative. (B) Primary spheres can be dissociated to form secondary spheres. (C) When cultured on plastic, A+Sca-1+ cells attach to plastic surface and maintain high levels of expression for vimentin (red). Nuclei are counterstained with DAPI (blue). (D, E) In attachment culture condition, A+Sca-1+ cells express GFAP (green) (D) and SMA (green) (E). (F-I) A+Sca-1+ cells have multilineage differentiation potential. (F) Under adipogenic condition, A+Sca-1+ cells are positive for Oil Red O. (G) A+Sca-1+ cells differentiate towards osteoblast, positive for Osteroportin (red). (I) A+Sca-1+ cells are positive for chondrocyte marker Collagen2 under chondrogenic differentiation condition.

3.3.4 Aldefluor (+) Sca-1 (+) cells are not derived from *Pdx1* lineage

The characteristics of A+Sca-1+ cells implied that they were mesenchymal cells. To explore the possibility that they might be of an epithelial origin, we took advantage of the *Pdx1-cre* (pancreatic and duodenal homeobox gene 1) lineage tracing mouse (Gu et al., 2002). *Pdx1* starts to express in the foregut region around Embryonic Day 8.5 (E8.5), and it marks the epithelia of pancreatic dorsal and ventral buds and part of the duodenal endoderm. In the homozygous *Pdx1* null mouse, only remnants of the pancreatic buds remain but they fail to proliferate (Offield et al., 1996). *Pdx1-cre* is expressed in the multipotent progenitor cells in the pancreas (Gu et al., 2002).

Pdx1-cre mice were crossed with transgenic mice carrying *Rosa26-loxp-CFP-loxp-mCherry* (a kind gift from Dr. Michael Wolfgang, Johns Hopkins). Following this cross, the progeny would be labeled with mCherry in all the pancreatic epithelial components, including acinar cells, ductal cells and endocrine cells; whereas the stromal components would be labeled with CFP. All the A+Sca-1+ cells were labeled with CFP, indicating they were derived from a mesenchymal cell source (Fig. 3.4).

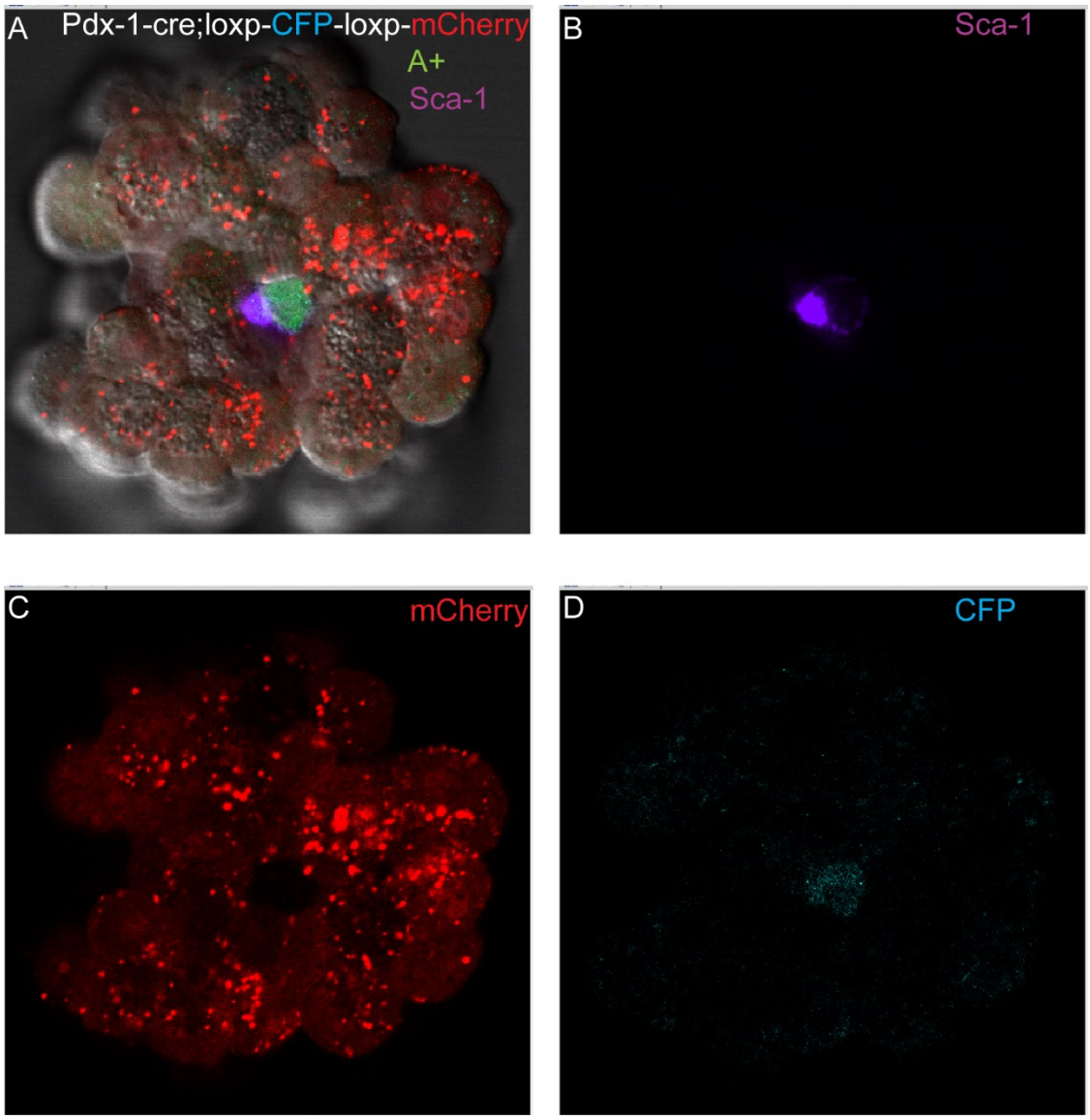


Figure 3.4: Aldefluor (+) Sca-1 (+) cells do not come from *Pdx1* lineage.
In the *Pdx1-cre; Rosa26-loxp-CFP-loxp-mCherry* mouse, all the pancreatic cells of epithelial lineage are labeled with mCherry (red); whereas the stromal components are labeled with CFP (blue). A+Sca-1+ cells overlap with CFP label, indicating their mesenchymal origin.

3.3.5 Aldefluor (+) Sca-1 (+) cells are upregulated upon cerulein induced acute pancreatitis

Cerulein is a decapeptide analogue (derived from Australian tree frog *Litoria caerulea*) of the pancreatic secretagogue cholecystokinin (CCK). Short-term cerulein administration leads to severe acute pancreatitis because of inappropriate intrapancreatic trypsinogen activation and subsequently, the autodigestion and death of the pancreatic cells (Mareninova et al., 2006). In the mouse pancreas, most exocrine cells are lost during this process, but in approximately 7 days following cerulein treatment, the pancreas regenerates almost completely (Siveke et al., 2008). Regenerating acinar cells reinstate genetic programs resembling those in the embryonic pancreatic precursors (Jensen et al., 2005). Cerulein induced pancreatitis is a widely used system to model human pancreatitis and to study pancreatic regeneration as well as the role of inflammation in the initiation of pancreatic cancer (Collins et al., 2012; Elsasser et al., 1986; Guerra et al., 2007; Jensen et al., 2005).

The abundance of A+Sca-1+ cells changed during the course of pancreatic regeneration after the administration of cerulein (Fig. 3.5). Under normal conditions, there could be observed at most one A+Sca-1+ cell per acinar unit. One day after the application of cerulein, the population of A+Sca-1+ cells were greatly increased. Gradually, the number of A+Sca-1+ cells returned to the baseline level in the course of approximately 7 days. The increase in abundance of the A+Sca-1+ cells during pancreatic injury and regeneration suggested that they might play a role during pancreatic remodeling, either through direct differentiation or through paracrine signaling, as discussed below.

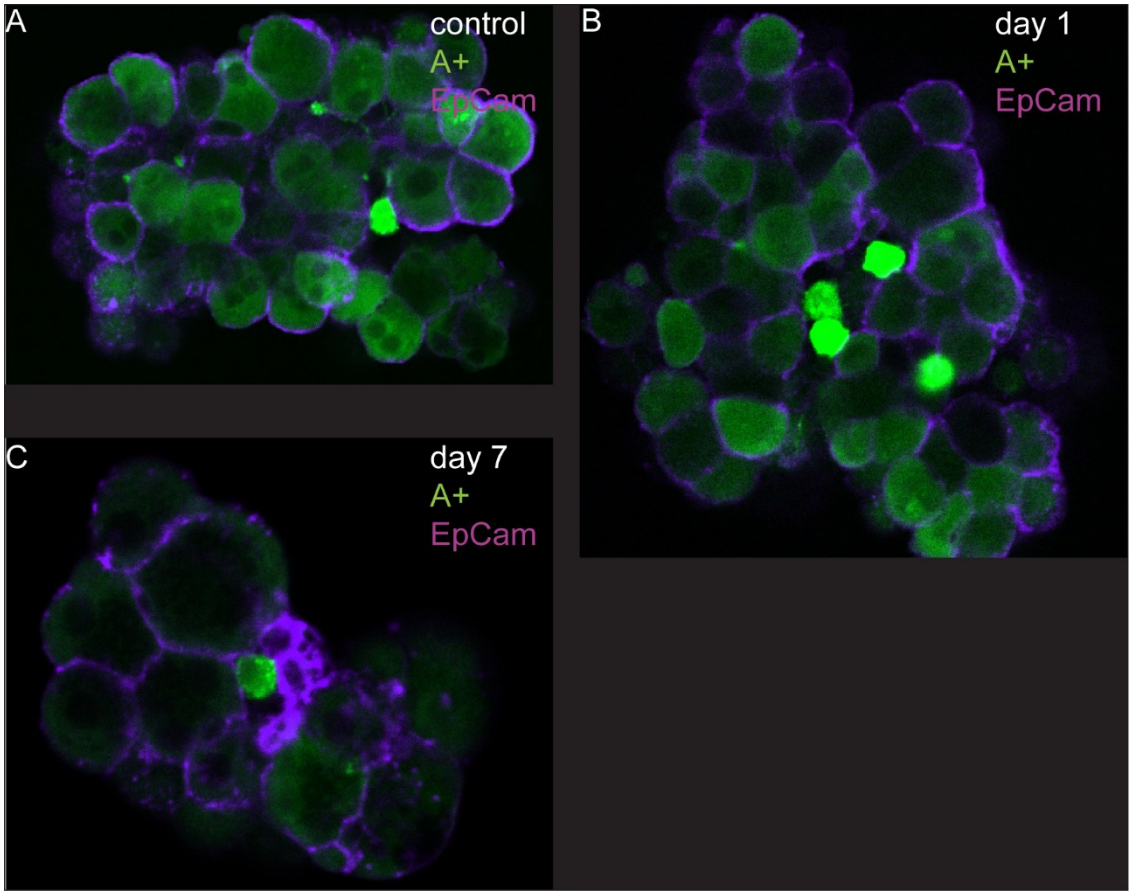


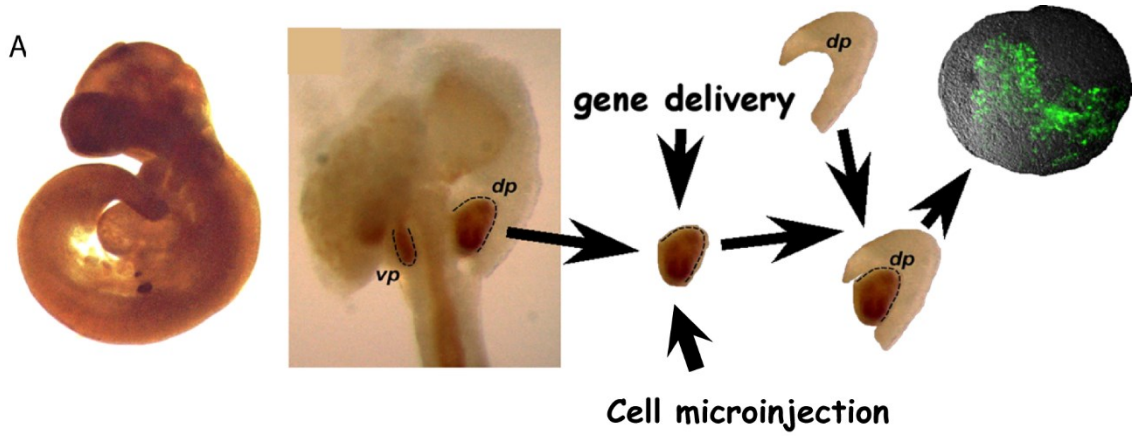
Figure 3.5: The number of Aldefluor (+) Sca-1 (+) cells increases during cerulein induced pancreatitis.

2 consecutive days of 6 doses of cerulein injections per day induce acute pancreatitis in mice. Acinar cells die but the pancreas resumes morphological and functional normality within 7 days. A+Sca-1+ population is upregulated during pancreatic regeneration. In all the panels, A+ cells are shown in bright green. EpCam, purple. (A) Control. (B) Day 1 after cerulein treatment. (C) Day 7.

3.3.6 Aldefluor (+) Sca-1 (+) cells contribute to exocrine and endocrine lineage ex vivo.

Dissected pancreatic dorsal buds from as early as E10.5 mouse embryos can be cultured *ex vivo (Esni et al., 2005; Gittes, 1993; Petzold and Spagnoli, 2012)*. Epithelium within the intact dorsal buds will undergo morphogenesis and cytodifferentiation to form both endocrine and exocrine tissue, recapitulating the endogenous developmental processes (Golosow and Grobstein, 1962; Miralles et al., 1998; Spooner et al., 1970). The *ex vivo* cultured embryonic mouse pancreas provide an appropriate microenvironment to investigate the differentiation potential of A+Sca-1+ cells isolated from adult mouse pancreas (Rovira et al., 2010).

A+ cells from mT/mG mice, with global expression of membrane-tagged tdTomato (Muzumdar et al., 2007), were flow sorted and microinjected into dissected E12.5 dorsal buds (Fig. 3.6A). Interestingly, under this condition, A+ cells were able to differentiate into both exocrine and endocrine cells (Fig. 3.6B, B' and C, C'). The differentiation of mesoderm derived population (A+ cells) into endoderm epithelial cell types (exocrine and endocrine cells), suggested that A+ cells could overcome their germ layer commitment (Grove et al., 2004). The plasticity of A+ cells makes them a potential source to, for example, derive new β cells.


A
dp
vp
gene delivery
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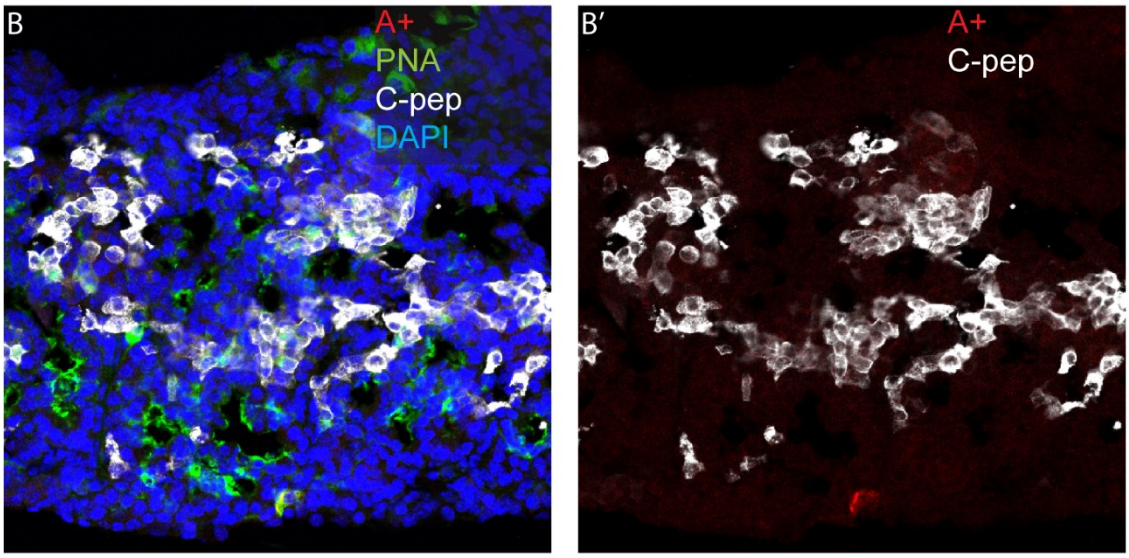
dp
Cell microinjection


B
A+
PNA
C-pep
DAPI
B′
A+
C-pep

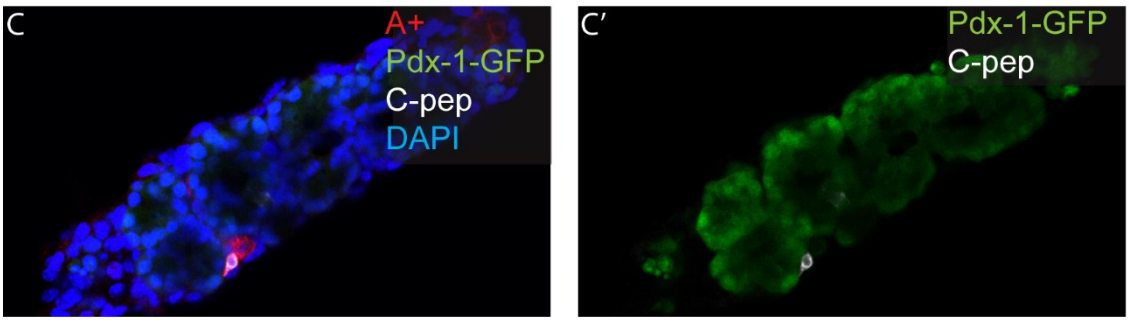

C
A+
Pdx-1-GFP
C-pep
DAPI
C′
Pdx-1-GFP
C-pep

Figure 3.6: Isolated Aldefluor (+) cells differentiate to endocrine and exocrine cells.

(A) Experiment outline. Dorsal buds from E12.5 mouse can be dissected out. In the figure pancreatic buds are labeled by *Pdx1 in situ* hybridization to indicate the relative location. Flow sorted A+ cells are injected into the dorsal buds, and subsequently, the buds can be cultured *in vitro*. Cultured buds will undergo morphogenesis and cellular differentiation, resembling the development process *in vivo*. (B, B') A+ cells (red) from an mT/mG donor express exocrine marker PNA (green). C-peptide (white) labels insulin secreting β-cells. DAPI, blue. (C, C') A+ cells (red) from mT/mG donor express β-cell marker C-peptide (white). Recipient bud has *Pdx1-GFP* transgene. A+ cells do not have GFP expression, implying that the observed differentiation event is not due to cell fusion.

3.3.7 Aldefluor (+) Sca-1 (+) cells secrete similar factors as embryonic mesenchyme

As previously mentioned, A+Sca-1+ cells had long cytoplasmic processes. This cellular feature is a characteristic for cells forming the niche, examples of which include distal tip cell in *C. elegans* germ line (Kimble and Crittenden, 2007) and niche cells in the *Drosophila* intestine (Mathur et al., 2010). Furthermore, the fact that A+Sca-1+ cells resemble mesenchymal stem cells indicates that they may also secrete paracrine signals (Chen et al., 2008b; Gnecchi et al., 2008). To explore this possibility, we carried out a cytokine array analysis (RayBiotech G3) to identify cytokines secreted by A+Sca-1+ cells. These cells were cultured on plastic. Conditioned media from the culture were collected and applied onto the cytokine array, together with media secreted from E12.5 pancreatic mesenchyme. Embryonic mesenchymal tissue has been shown to provide signals to induce the proper differentiation and morphogenesis of pancreatic epithelia (Attali et al., 2007; Duvillie et al., 2006; Gittes et al., 1996; Jiang et al., 1999). We observed that the cytokine profiles of the A+Sca-1+ secretome is very similar to that of the E12.5 pancreatic mesenchyme (Fig. 3.7).

Secreted component	Gene symbol	Pathway	A+/M+ (adult)	MEF^{panc} (E12.5)	Permissive sphere medium (mouse only)
Retinoic acid	-	RAR	Aldh1a1, Aldh1a2,	Aldh1a2	
FGF10/FGF7	*Fgf10*	PI3K/Akt; MAPK	Yes, FgF10	Yes, Fgf10	bFGF (20 ng/ml)
Wnt5a	*Wnt5a*	Fzd/PCP	Yes	Yes	
Activin A	*Inhba*	Actr/Smad	no	Yes	
BMP3b	*Gdf10*	Bmpr/Smad	Yes	no	
BMP4	*Bmp4*	Bmpr/Smad	Yes	no	
Igf1	*Igf1*	PI3K/Akt; MAPK	Yes	Yes	Note: EGF (20 ng/ml)
Igf2	*Igf2*	PI3K/Akt; MAPK	no	Yes	
Hedgehog	*Dhh*	Smo/Ptch/Gli	Yes (low)	no	
Il11	*Il11*	Gp130-JAK-Stat	no	Yes	
Il6	*Il6*	Gp130-JAK-Stat	Yes	no	
LIF	*Lif*	Gp130-JAK-Stat	no	no	LIF:10 ng/ml
Il17d	*Il17d*	Il17r family	Yes	Yes	
Sdf1	*Cxcl12*	Cxcr4	no	Yes	
Cxcl13	*Cxcl13*	Cxcr5/Cxcr2	Yes	no	
CTGF	*Ctgf*	HSPG/ECM	Yes	Yes	
VEGF	*Vegfa*	Vegfr/Flt family	no	Yes	
PGE2, PGI2	-	Ptger-family	Ptges3 (low) Ptgis high	Ptges3, Ptgis high	Note: N2 supp + B27

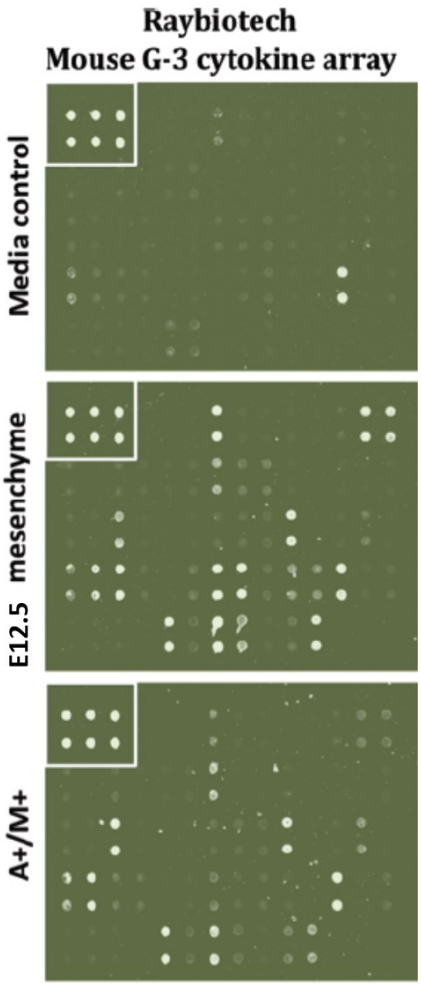


Figure 3.7: Cytokine array

(Meritxell Rovira).

A+Sca-1+ cells secrete similar soluble factors as E12.5 pancreatic mesenchymal cells.

3.4 Discussion

In summary, we identified a novel population of A+Sca-1+ cells in the adult mouse pancreas. The characteristics of these cells include: (1) they are located next to blood vessels and have long cytoplasmic processes; (2) they form mesenchymal spheres *in vitro* and display features of mesenchymal stem cells; (3) they originate from a non-epithelial lineage; (4) they are upregulated during cerulein induced acute pancreatitis; (5) they can be induced to differentiate into both exocrine cells and endocrine cells *ex vivo* and (6) they secrete paracrine signals.

In the future, it would be desirable to develop a differentiation protocol *in vitro* and to characterize the functions of this A+Sca-1+ population *in vivo*. I showed that A+Sca-1+ cells were proliferative in culture and they could differentiate into an epithelial lineage within the pancreatic dorsal buds. Further dissection of the inductive signals from the embryonic buds would guide us in designing culture conditions to mimic this environment, and possibly to coerce these A+Sca-1+ cells towards the endocrine β-cell fate *in vitro*. Studies have shown that tissue-derived stem cells retain epigenetic memories from the cell of origin, thus are more likely to differentiate back into the tissue cell type of origin (Kim et al., 2010; Polo et al., 2010). As a result, A+Sca-1+ cells might be more amenable to differentiation into β cells than both undifferentiated embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which are mainly the two cell types currently being evaluated to generate new β cells (Assady et al., 2001; D'Amour et al., 2006; Kroon et al., 2008; Zhang et al., 2009).

Meanwhile, the endogenous functions of A+Sca-1+ cells need to be considered. The mesenchymal features of these cells seem to imply that they may play a role during lipid deposition and stromal infiltration during pathological changes of the pancreas. Microarray and qRT-PCR results showed that A+Sca-1+ cells expressed high level of Aldh1a2, Axin2, vimentin, and GFAP (data not shown). All these markers, however, are expressed in other cell types inside the pancreas. Further efforts to identify a unique marker for A+Sca-1+ cells are warranted in order to generate a lineage tracing mouse line to study the *in vivo* function of this population.

In addition, the fact that A+Sca-1+ cells secrete similar factors as embryonic mesenchyme, locate adjacent to blood vessel and display long cytoplasmic processes make them a likely candidate to constitute the niche microenvironment for pancreatic tissue-resident stem cells. Further efforts to dissect the potential paracrine signaling pathway would presumably involve small molecule inhibitors and gene knock-out/knock-down. A+Sca-1+ cells may also be explored as a supporting or paracrine source for the derivation of new β cells.

Chapter 4. *Dicer* is required for maintenance of adult pancreatic acinar cell identity and plays a role in Kras-driven pancreatic neoplasia

4.1 Introduction

The microRNA pathway is a critical regulator of gene expression, and Dicer, an RNase III-containing enzyme, is a central component of the microRNA (miRNA) processing machinery. Deregulation of miRNAs has been implicated in a variety of biological process including cancer (Carthew, 2006), (Sevignani et al., 2006). In mice, global loss of *Dicer* leads to lethality before embryonic day 7.5 (Bernstein et al., 2003). Conditional inactivation of *Dicer* in numerous cell types from tissues such as the immune system (Cobb et al., 2005), retina (Bernstein et al., 2003), cortex, hippocampus (Davis et al., 2008) and ovary (Lei et al., 2010), has proven the essential function of *Dicer* in a number of tissue-specific contexts.

Previous work has shown that miRNA levels fluctuate during cancer initiation and progression. Both tumor suppressor and oncogenic miRNAs have been described (Chang and Mendell, 2007; Zhang et al., 2007). In general, tumor cells display a global downregulation of miRNA expression (Lu et al., 2005). Recently, several reports have indicated that *Dicer* may function as a haploinsufficient tumor suppressor, as heterozygosity for *Dicer* enhances tumor development, whereas enforced *Dicer* homozygous deletion causes inhibition of tumorigenesis in certain tumor models (Kumar et al., 2009; Lambertz et al., 2010; Zhang et al., 2013).

Recent publications have shown that pancreatic acinar cells display unexpected plasticity, and are capable of undergoing dramatic changes in differentiation in the settings

of both chronic pancreatitis and pancreatic cancer. Lineage tracing approaches have provided evidence that acinar cells in the adult pancreas have the potential to transdifferentiate into metaplastic ductal cells (Strobel et al., 2007). This acinar-to-ductal metaplasia (ADM) event precedes the formation of pancreatic intraepithelial neoplasia (PanIN) (Means et al., 2005; Reichert and Rustgi, 2011; Shi et al., 2013; Zhu et al., 2007). Acinar cells have similarly been shown to be an effective cell-of-origin for $Kras^{G12D}$-induced PanIN and pancreatic ductal adenocarcinoma (PDAC) (De La et al., 2008; Guerra et al., 2007; Habbe et al., 2008).

Deletion of *Dicer* at the onset of pancreatic development results in defects in all pancreatic lineages (Lynn et al., 2007), while loss of *Dicer* in the pancreas at a late gestational stage (E18.5) results in defects in acinar cell differentiation and morphogenesis (Prevot et al., 2013). Interestingly, the adult pancreas seems to be extremely sensitive to changes in the abundance of *Dicer* (Morita et al., 2009). In the *Dicer* hypomorphic mouse created by Morita et al (Morita et al., 2009), *Dicer* expression was reduced to 20% in all tissues and the only observed abnormality was in the adult pancreas.

The motivation of the present study was to examine the role of miRNAs in normal pancreatic tissue homeostasis and during the initiation and progression of pancreatic cancer. Instead of investigating individual miRNAs, we deleted the miRNA master regulator *Dicer* in the adult exocrine pancreas with or without simultaneous activation of oncogenic $Kras^{G12D}$. This experiment was designed to provide initial insights into the role of the miRNA pathway as a whole, as well as revealing the dominant mechanism of regulation. We found that in normal pancreata, deletion of both copies of *Dicer* resulted in

the progressive loss of differentiated acinar cells, associated with the initiation of epithelial-to-mesenchymal transition (EMT). These changes were additionally accompanied by ADM and pancreatic fibrosis. In concert with *Kras*^{G12D} activation, homozygous *Dicer* deletion accelerated ADM formation, but not PanIN initiation. In contrast, PanIN formation was enhanced in the setting of *Dicer* haploinsufficiency. Regardless of genotype, PanIN lesions were observed to have retained Dicer expression. Thus, in addition to being required for the maintenance of acinar cell identity, fractional changes in Dicer gene activity may have significant impact on the pancreatic response to oncogenic stimuli.

Contributions: The following chapter represents the collaborative work of Dr. Bidyut Ghosh and myself. Dr. Bidyut Ghosh was involved in designing the experiment and interpreting the data. He contributed Fig. 4.5. The rest of the work was completed by myself.

4.2 Materials and Methods

4.2.1 Mouse lines

Dicer$^{fl/fl}$ mice were crossed with *Mist1*$^{CreERT2/+}$ mice (gift from Stephen F. Konieczny**,** University of Indiana). An additional *lox-stop-lox Rosa26 YFP* allele (hereafter referred to as *LSL-YFP*) was incorporated as a lineage marker. The resulting *Mist1*$^{CreERT2/+}$*; LSL-YFP*; *Dicer*$^{fl/wt}$ mice were further intercrossed or outcrossed onto a conditional *LSL-Kras*^{G12D} allele, as previously described (Habbe et al., 2008). Mice were euthanized by cervical dislocation under Isoflurane anesthesia. There was no surgery performed and animals exhibiting discomfort were euthanized. All procedures were performed under the

approval of the Johns Hopkins University School of Medicine Animal Care and Use Committee guidelines (IACUC, protocol number M012M353).

4.2.2 Tamoxifen induced *Dicer* deletion

Induction of CreERT2 activity was initiated at 8 weeks of age by administering 5mg of tamoxifen (Sigma, 10540-29-1) for 3 consecutive days via intra-peritoneal (IP) injections. Control littermates were injected with corn oil alone.

4.2.3 Pancreatic acinar isolation and *in vitro* culture

Acinar units were isolated as described previously (Esni et al., 2005). Briefly, mouse pancreata were perfused from common bile duct with 0.375 mg/ml Collagenase-P (Roche, 11213857001) in Hank's balanced salt solution (HBSS). Subsequently, pancreata were harvested and incubated in 37°C waterbath for 11 mins. Following multiple washes with HBSS, 5% FBS, the resulting acini were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Invitrogen), soybean trypsin inhibitor (Sigma) and antibiotics in low attachment 24-well plates (Corning). Acini were treated with either DMSO or with 4-hydroxytamoxifen (Sigma, H7904) at 20 ng/ml for 5 consecutive days to induce Cre-mediated deletion of *Dicer in vitro*.

4.2.4 Immunohistochemistry and immunofluorescence

Pancreata were harvested at indicated time points and were fixed in 4% paraformaldehyde, followed by standard paraffin or OCT embedding. Tissue was then cut into 5-10 μM sections. Incubations with primary antibodies were performed overnight at 4°C using standard techniques in PBS containing 0.2% Triton and 10% FBS using the

following antibodies or probes: rat anti-CD49f (BD Biosciences 555734, 1:100), phalloidin (Invitrogen R415, 1:500), rat anti-Ecad (Invitrogen 131900, 1:200), rabbit anti-Ki67 (Abcam Ab16667, 1:50), rabbit anti-Sox9 (Millipore ab5535, 1:200), chicken anti-Vimentin (Millipore ab5733, 1:500), rat anti-Ncad (Abcam Ab12221, 1:200), rat anti-EpCam (Biolegend, 118214, 1:100), rabbit anti-Amalyse (Sigma A8273, 1:500), DBA (Vector Labs AS2034, 1:200), rabbit anti-Dicer (Clonegene CG031, 1:1000). For immunohistochemistry, HRP-conjugated secondary antibodies were obtained from Vector Labs (MP-7401). 3-3-Diaminobenzidine tetrahydrochloride (Vector Labs) was used as a chromogen. Bright-field images were acquired using an Olympus BX40 light microscope. For immunofluorescence, secondary antibodies were obtained from Jackson ImmunoResearch and used at 1:300 dilution. Samples were mounted with Fluorescence Mounting Medium (Dako, S3023). Slides were imaged on the Nikon A1 confocal microscope system.

4.3 Results

4.3.1 *Dicer* knockout in mature pancreatic acinar cells

In order to investigate the role of *Dicer* in the maintenance of adult acinar cell differentiation, we generated *Mist1*^{CreERT2}*; Dicer*$^{fl/fl}$*; LSL-YFP* mice (Habbe et al., 2008). Tamoxifen was administered to 8-week-old adult mice via intraperitoneal injection. We followed morphological changes in pancreata up to 6 months after tamoxifen injection (Fig. 4.1A). Successful Cre-mediated recombination could be visualized by the expression of YFP in more than 90% of the acini (Fig. 4.1B, C) and the expression of YFP was highly correlated with the elimination of Dicer protein, as assessed by Dicer

immunohistochemistry (Fig. 4.1D, E). During the time course of this study, the most extensive pancreatic abnormalities were observed at one month following the deletion of *Dicer* (Fig. 4.1F-K, Fig. S4.1). In some cases, pancreatic lobes were characterized by extreme acinar cell atrophy, with only remnants of ductal like structures left behind (Fig. 4.1I). This was accompanied by a prominent inflammatory infiltrate (Fig. 4.1I). In the most severe scenario, areas of fibrosis were observed to replace the majority of normal pancreatic acinar tissue (Fig. S4.1A, B). By six months post-tamoxifen administration, the histology of pancreata resumed normality with little sign of tissue damage (Fig. 4.1K). Noticeably, at six months after the deletion *of Dicer***,** regions of YFP expression became more sporadic (Fig. S4.1, Fig S4.2). We interpreted this increasingly patchy YFP expression as evidence of expanding pancreatic exocrine tissue originating from acinar cells that had escaped *Dicer* deletion. However, we cannot exclude other possibilities such as a transdifferentiated and/or a progenitor cell population contributing to the regenerated pancreata. In fact, we observed a general proliferative response even in non-acinar tissue after *Dicer* deletion (Fig.4.4B, Fig. S4.2). Notably, the histology of the pancreata from Mist1^{creERT2}; *Dicer*$^{fl/wt}$ animals remained normal across all time points (Fig. 4.1G).

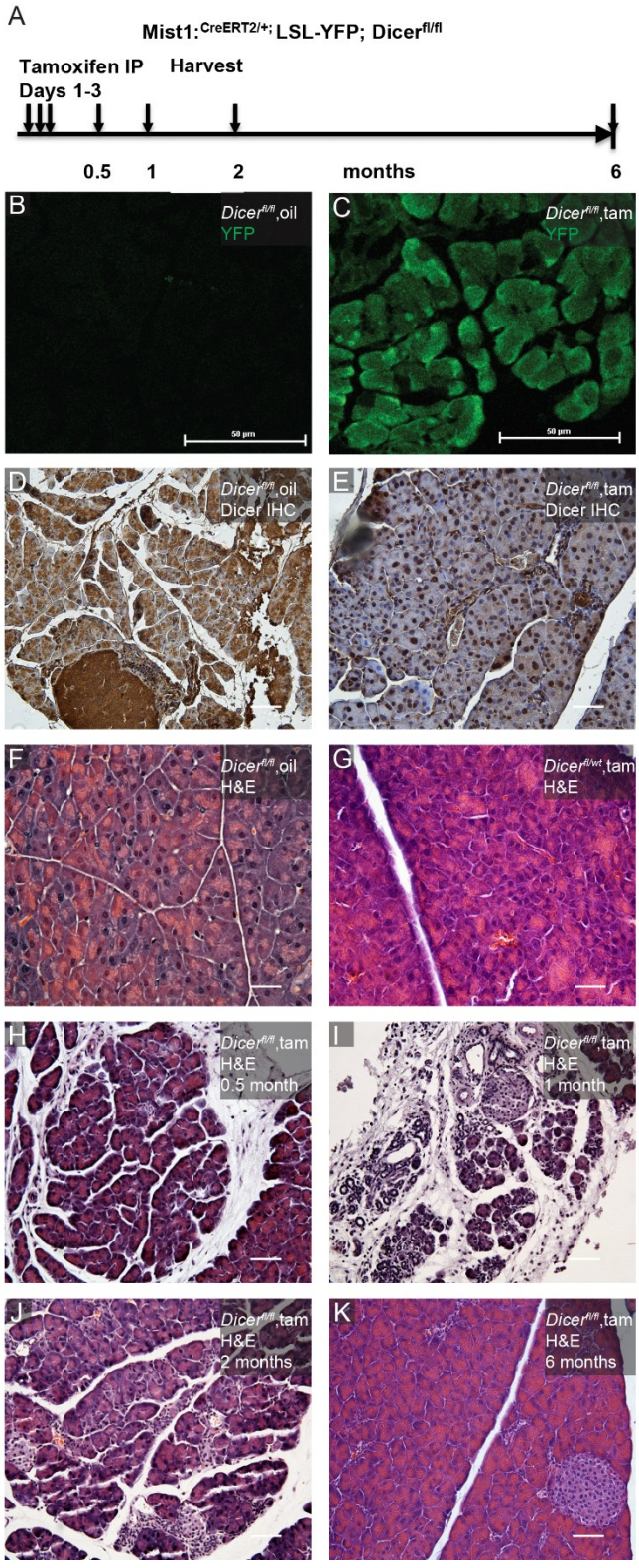

A
Mist1:CreERT2/+; LSL-YFP; Dicerfl/fl
Tamoxifen IP
Days 1-3
Harvest
0.5
1
2
months
6
B
Dicerfl/fl,oil
YFP
C
Dicerfl/fl,tam
YFP
D
Dicerfl/fl,oil
Dicer IHC
E
Dicerfl/fl,tam
Dicer IHC
F
Dicerfl/fl,oil
H&E
G
Dicerfl/wt,tam
H&E
H
Dicerfl/fl,tam
H&E
0.5 month
I
Dicerfl/fl,tam
H&E
1 month
J
Dicerfl/fl,tam
H&E
2 months
K
Dicerfl/fl,tam
H&E
6 months

Figure 4.1: Deletion of *Dicer* alters histology in exocrine pancreas.
(A) Experimental regimen. Tamoxifen injection was performed on 6-8 weeks old mice. (B) Pancreas treated with corn oil has no YFP expression. (C) Widespread Cre-mediated recombination is shown by YFP expression following tamoxifen induction. (D, E) Immunohistochemistry for Dicer on *Mist-Cre^{ERT2}; LSL-YFP; Dicer$^{fl/fl}$* mice treated with corn oil only (D) or those treated with tamoxifen (E). There is almost complete loss of cytoplasmic Dicer signal in the acinar cells in tamoxifen-treated tissue. Residual nuclear signal likely represents non-specific antibody labeling. (F-K) H & E staining showing the time-course of histologic change after tamoxifen administration. (F) *Dicer$^{fl/fl}$* oil control. (G) *Dicer$^{fl/wt}$*. Tissue is morphologically indistinguishable from oil control. (H) *Dicer$^{fl/fl}$* 0.5 month after tamoxifen injection. (I) *Dicer$^{fl/fl}$* 1 month after tamoxifen injection. Tissue changes are most dramatic at this time point. (J) 2 months. (K) 6 months. At this time point, pancreas from Dicer knockout mice is nondistinguishable with wildtype. Scale bars depict 50 microns.

4.3.2 *Dicer* is required for the maintenance of mature acinar cell identity

We sought to characterize changes occurring following acinar cell-specific *Dicer* deletion in additional detail. We used two markers of cell polarity, CD49f (integrin subunit α6) and phalloidin. CD49f forms a heterodimer with integrin β1 subunit and is present on the basal membrane of pancreatic acini (Bombardelli et al., 2010); while phalloidin stains apical actin web concentrated at the apical side of acinar cells. Using immunoflourescent staining, we found that these polarity markers were mislocalized in the acinar cells of the *Dicer* knockout. CD49f translocated to the lateral membrane (Fig. 4.2) and the expression level of CD49f was significantly increased compared with control acini. Phalloidin showed a diffuse pattern of staining in the *Dicer*-deleted animals, rather than labeling apically-oriented actin filaments (Fig. 4.2), indicating a loss of cell polarity.

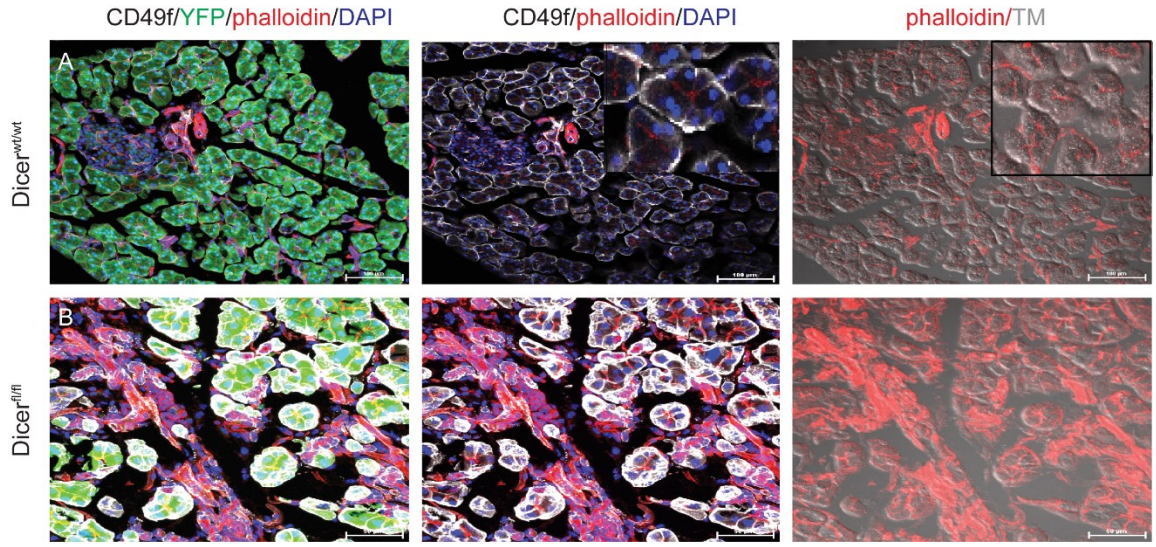


Figure 4.2: Deletion of Dicer induces loss of polarity in acinar cells following Dicer deletion.
(A) Epithelial cell polarity marker CD49f (white) is expressed basally, while phalloidin labeling (red) is observed apically in control *Mist-Cre^{ERT2}; LSL-YFP; Dicer$^{wt/wt}$* pancreas following tamoxifen treatment. (B) In the *Mist-Cre^{ERT2}; LSL-YFP; Dicer$^{fl/fl}$* mice, *Dicer* deletion leads to translocation of CD49f in the lateral membrane, and loss of apical phalloidin labeling. Cytoplasmic expression of YFP is shown in green as a surrogate marker of Cre-mediated recombination. Nuclei are stained with DAPI (blue).

Following *Dicer* deletion, pancreatic acinar cells also lost their classic rosette-like epithelial organization (Fig. 4.2B, Fig. 4.3B, D). This phenotype was similar to what was observed by Morita et al (Morita et al., 2009) in the *Dicer* hypomorphic pancreas. In addition, acinar cells lacking Dicer developed a partial mesenchymal phenotype. This process of EMT was documented by decreased expression of the epithelial marker Ecad and EpCam and increased expression of the mesenchymal markers Vimentin and Ncad (Fig. 4.3).

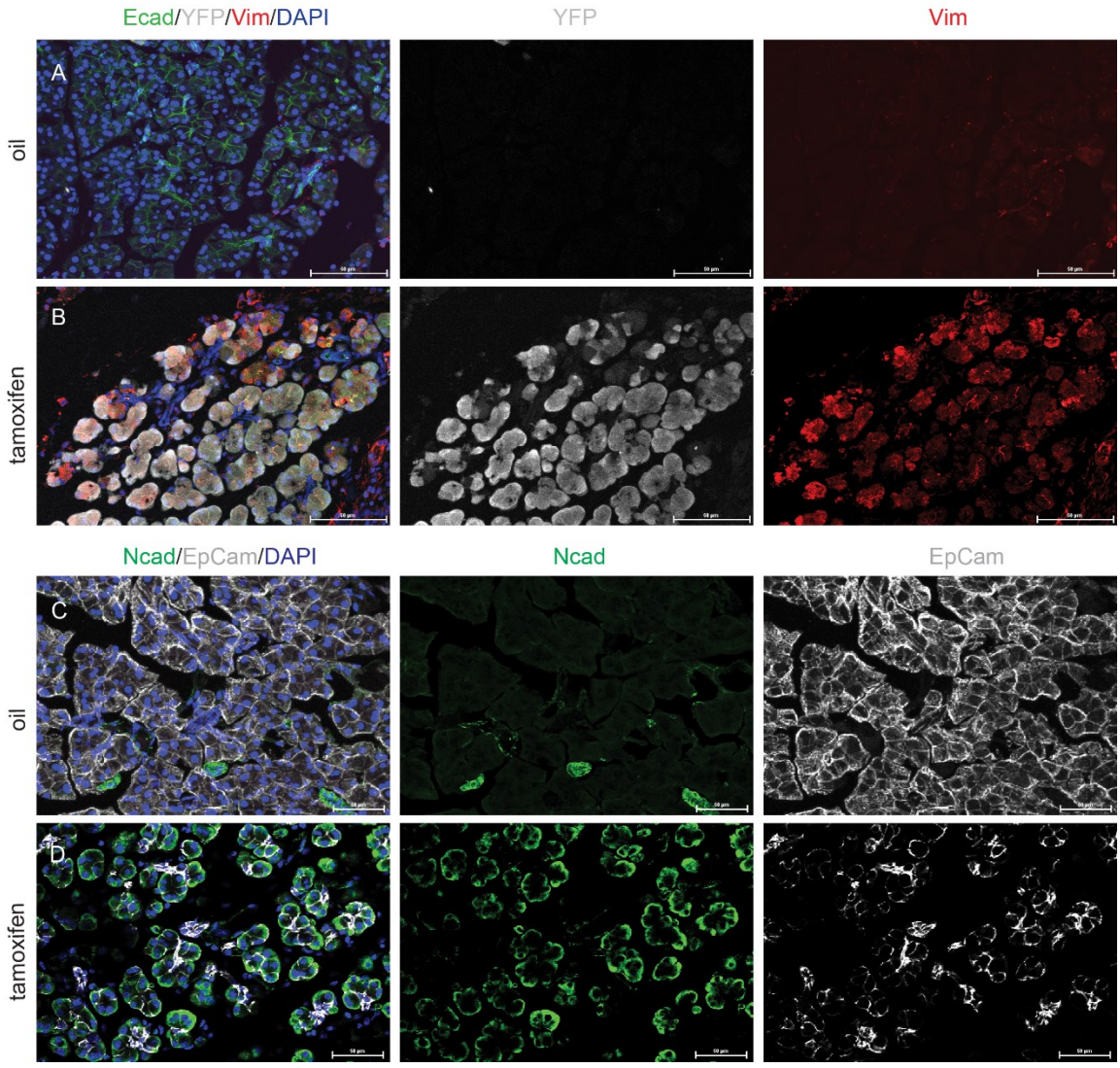


Figure 4.3: Adult acinar cells undergo epithelial to mesenchymal transition.
(A) Pancreata from oil injected *Mist-Cre^{ERT2}; LSL-YFP; Dicer$^{fl/fl}$* mice do not express YFP (white) or vimentin (red). Ecad (green) outlines acinar cells. (B) In tamoxifen treated *Mist-CreERT2; LSL-YFP; Dicer$^{fl/fl}$* mice, acinar cells show co-localization of YFP and vimentin. Ecad expression is reduced. (C, D) Representative immunofluorescent staining for the mesenchymal marker Ncad (green). (C) In oil treated control pancreas, acini lack any Ncad expression. (D) In tamoxifen-treated *Dicer$^{fl/fl}$* pancreas, there is membrane expression of Ncad. Note that there is simultaneous loss of EpCam expression (white), in *Dicer* deleted acini. Nuclei are stained with DAPI (blue).

Furthermore, in the absence of *Dicer*, some of the acinar cells showed evidence of ADM (Fig. 4.4). This was indicated by appearance of the YFP positive lineage marker in some of the duct-like structures, which were labeled by the lectin Dolichos Biflorus Agglutinin (DBA) as well as an antibody against Sox9 (Fig. 4.4, Fig. S4.1C, D). Following *Dicer* deletion, proliferative responses were observed in both acinar and nonacinar cells, presumably as a result of signals from pancreatic injury (Fig. 4.4A, B). An extensive quantification of Ki67 positive cells following tamoxifen administration revealed that maximum cellular proliferation occurred in the acinar cells at one month after loss of *Dicer*, with less frequent proliferation observed in other components of pancreas including ducts, islets and stroma (Fig. S4.2).

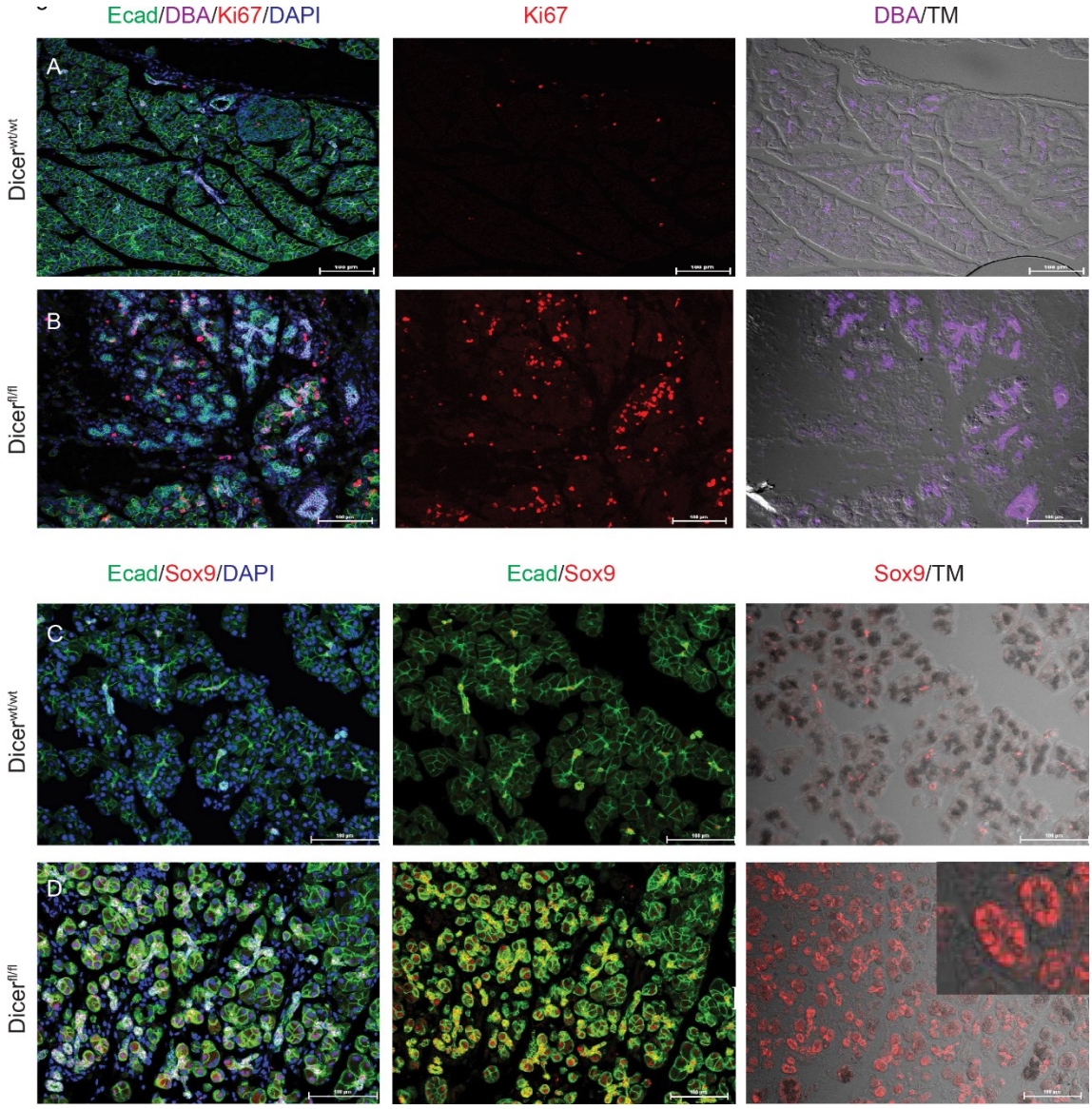


Figure 4.4: *Dicer* deletion leads to upregulation of ductal markers in the acinar population.

(A, B) Pancreatic ductal marker Dolichos Biflorus Agglutinin (DBA) (purple) is upregulated along with cell proliferation marker Ki67 (red) in the *Mist-Cre^{ERT2}; Dicer$^{fl/fl}$* mice following tamoxifen injection (B) compared to control *Mist-Cre^{ERT2}; Dicer$^{wt/wt}$* pancreata (A). (C, D) Sox9 (red), a duct-specific transcription factor, is also upregulated in acinar cells and in ADM lesions in *Mist-Cre^{ERT2}; Dicer$^{fl/fl}$* pancreata (D), while Sox9 is expressed only in ductal epithelial cells in control *Mist-Cre^{ERT2}; Dicer$^{wt/wt}$* pancreata (C). Epithelial marker Ecad is in green and nuclei are stained with DAPI (blue).

In summary, we observed a major identity shift in the acinar population upon homozygous *Dicer* knockout. Acinar cells lost apical-basal polarity and transdifferentiated into ductal or mesenchymal cell types, suggesting that Dicer is required for the maintenance of acinar cell differentiation.

4.3.3 *In vitro* acinar cultures confirm a cell autonomous effect of *Dicer* deletion

In the *Dicer* knockout pancreas, an activated inflammatory response is observed (Fig. 4.1). Based on the fact that morphological changes in pancreatic acinar cells can be induced by pancreatic inflammation alone (Liou et al., 2013; Strobel et al., 2007), we endeavored to assess whether the phenotypes we observed after *Dicer* deletion in acinar cells were a primary effect of *Dicer* knockout or a secondary effect of tissue injury. For these studies, we utilized an *in vitro* acinar culture system. In this setup, the macrophages, neutrophils and other components of the immune system were absent. Isolated acini from *Mist1*$^{CreERT2/+}$*; LSL-YFP; Dicer*$^{fl/fl}$ mice were handpicked and cultured in the presence of 4-hydroxytamoxifen (4-OH) or DMSO control. Acini incubated with 4-OH showed down-regulation of Ecad (Fig. 4.5A, K vs. 4.5F, P) and up-regulation of Vimentin (Fig. 4.5L vs. 4.5Q); similar to what we observed *in vivo* (Fig. 4.3). Acini incubated with DMSO control maintained their epithelial identity (Fig. 4.5K, L, M, N, O). In these experiments, YFP expression following 4-OH treated acini was used as a surrogate marker for successful deletion of *Dicer* (Fig. 4.5C, H, M, R). These studies demonstrate a cell autonomous requirement for *Dicer* in the maintenance of acinar cell identity.

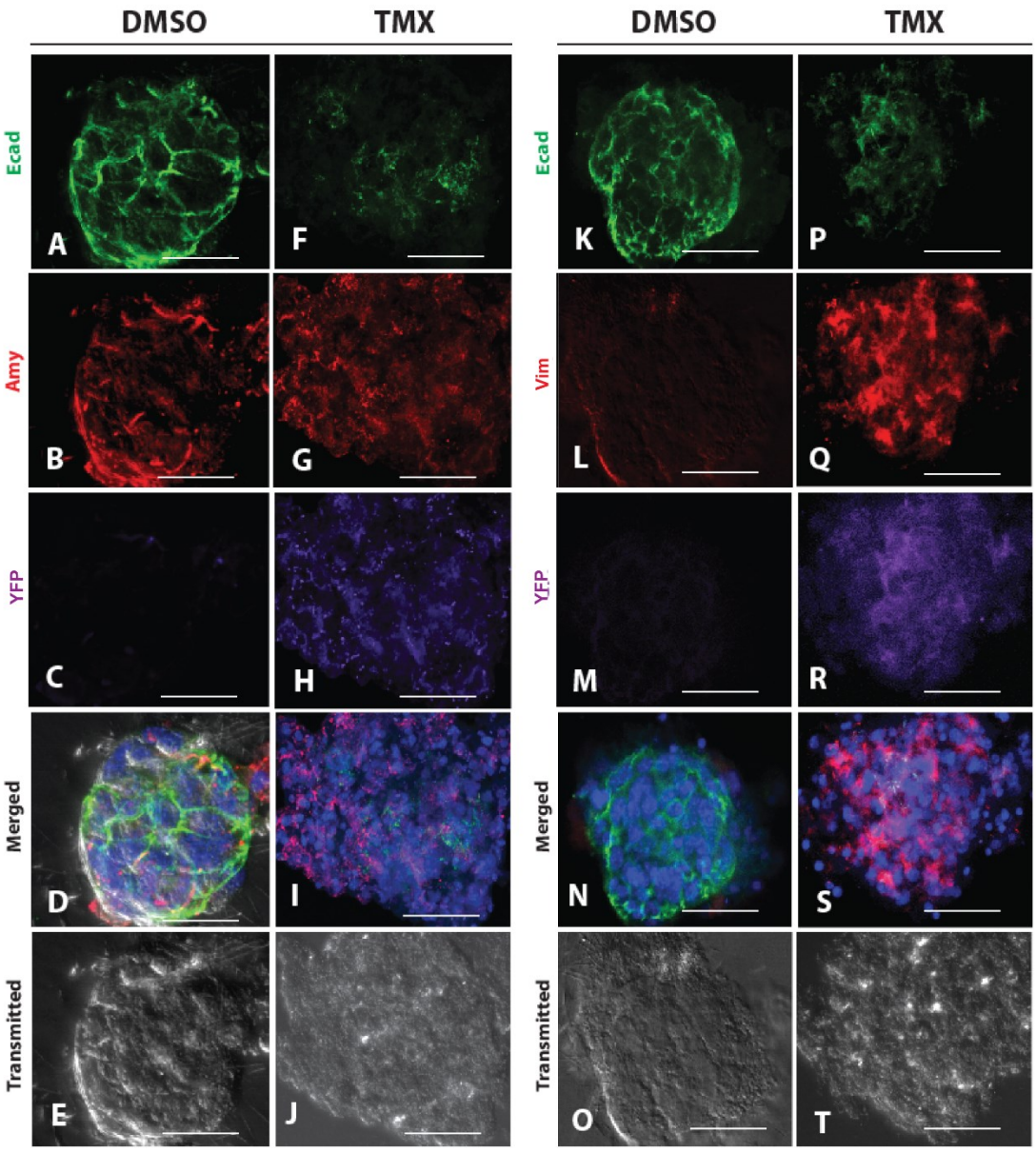

DMSO
TMX
DMSO
TMX
Ecad
Amy
YFP
Merged
Transmitted
Ecad
Vim
YFP
Merged
Transmitted
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T

Figure 4.5: *In vitro* deletion of *Dicer* in pancreatic acinar cells results in initiation of EMT.

(A-E) and (K-O) depict multi-channel fluorescent imaging of two representative pancreatic acini independently harvested from *Mist1*$^{CreERT2/+}$*;LSL-YFP; Dicer*$^{fl/fl}$ mice and treated with DMSO control. (F-J) and (P-T) depict multi-channel fluorescent imaging of two representative pancreatic acini isolated from *Mist1*$^{CreERT2/+}$*;LSL-YFP; Dicer*$^{fl/fl}$ mice and treated with tamoxifen (TMX) to induce Cre activity. **(**A-J), Immunostaining of isolated acinus with Amylase (red), Ecad (green) and GFP (purple). There is no change in Amylase expression following 5 days incubation with either DMSO control (A-E) or tamoxifen treatment (F-J). But significant down-regulation of Ecad can be observed. (K-T), Immunostaining of isolated acini with Vimentin (red), Ecad (green) and YFP (purple). Downregulation of Ecad is accompanied by activation of vimentin in tamoxifen-treated cells (P-T) compared to DMSO control (K-O). Activation of YFP in (H) and (R) document tamoxifen-induced Cre-activity.

4.3.4 *Dicer* haploinsufficiency enhances PanIN formation in an oncogene-dependent manner

Loss of *Dicer* in normal adult pancreatic acinar tissue resulted in the initiation of ADM, which is a proposed precursor of PanIN (Brune et al., 2006; Reichert and Rustgi, 2011; Shi et al., 2013). We therefore sought to determine whether loss of *Dicer* would facilitate PanIN initiation. Recent studies have shown that the *Mist1*$^{CreERT2/+}$*; LSL-Kras*^{G12D} mouse model fully recapitulates the initiation and progression of early human pancreatic neoplasia (Habbe et al., 2008). To test the function of Dicer in the context of oncogenic Kras-driven pancreatic neoplasia, we crossed *Dicer*$^{fl/fl}$ mice onto the *Mist1*$^{CreERT2/+}$*; LSL-Kras*^{G12D} mice. We generated *Mist1*$^{CreERT2/+}$*; LSL-Kras*^{G12D}; *Dicer*$^{fl/fl}$ (*Dicer-floxed-Kras*) and *Mist1*$^{CreERT2/+}$*; LSL-Kras*^{G12D}; *Dicer*$^{fl/+}$ (*Dicer-het-Kras*) mice, and compared them with *Mist1*$^{CreERT2/+}$*; LSL-Kras*^{G12D}; *Dicer*$^{wt/wt}$ (*Dicer-wt-Kras*) mice. Interestingly, immunohistochemistry for Dicer in the pancreata of *Dicer-wt-Kras* mice demonstrated a tissue-wide decrease of Dicer protein (Fig. 4.6A vs B). This observation is in line with the hypothesis that the global down-regulation of miRNA expression in tumors may be driven by alterations in the expression levels or activity of Dicer (Martello et al., 2010). Notably, in the same sections, PanIN lesions displayed higher levels of *Dicer* expression (Fig. 4.6B, arrows). In the *Dicer-floxed-Kras* mice, there was an almost complete loss of cytoplasmic Dicer protein in the acinar compartment, although some nuclear Dicer staining remained, likely representing nonspecific signal (Fig. 4.6C). Strikingly, the vast majority of metaplastic and neoplastic lesions preserved a moderate level of Dicer protein (Fig. 4.6D), suggesting selective pressure to retain at least some level of Dicer expression during the

initiation of pancreatic neoplasia. This is consistent with recent work showing certain types of tumors select against full loss of Dicer function (Kumar et al., 2009).

We quantified the pancreatic phenotypic abnormalities, including ADM and PanIN, in both the *Dicer-floxed-Kras* and *Dicer-het-Kras* mice. We observed that *Dicer-het-Kras* mice developed PanINs at earlier time points and at much higher frequency than the *Dicer-floxed-Kras* mice and the *Dicer-wt-Kras* mice in the same cohort (Fig. 4.6E-H). At the two-month time point, *Dicer-het-Kras* mice had PanIN comprising approximately 4% of total pancreatic surface area, compared to the 0.8% in *Dicer-floxed–Kras* mice and 1% in *Dicer-wt–Kras* mice (Fig. 4.6H). In contrast, *Dicer-floxed-Kras* mice have an increase in ADM compared with *Dicer-het-Kras* and *Dicer-wt-Kras* pancreata. (Fig. 4.6H). This is consistent with our previous assumption, indicating that *Dicer* loss accelerates the formation of ADM under conditions of Kras activation, but in the absence of Dicer these ADM lesions are unable to further progress to PanIN.

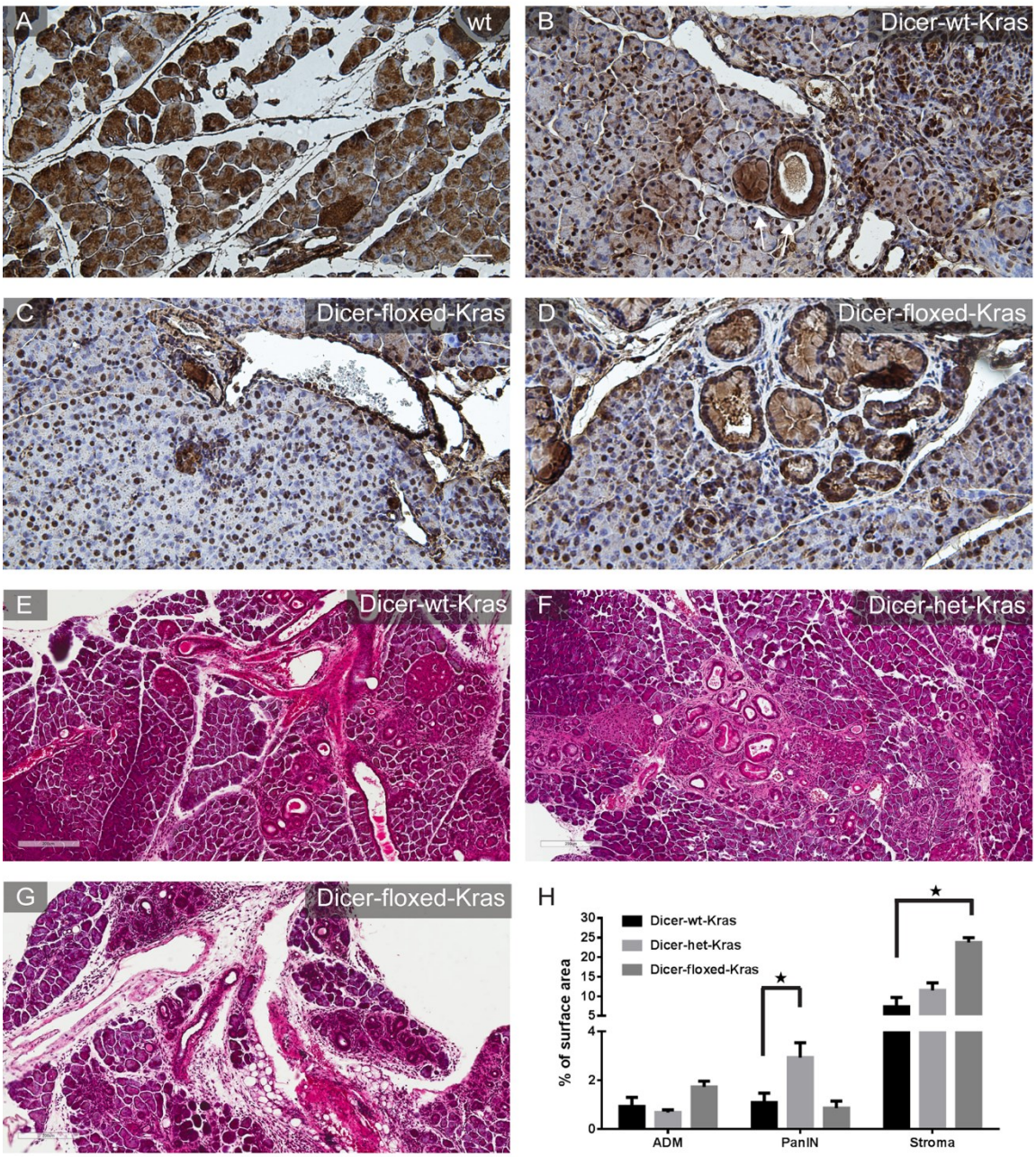

A
wt
B
Dicer-wt-Kras
C
Dicer-floxed-Kras
D
Dicer-floxed-Kras
E
Dicer-wt-Kras
F
Dicer-het-Kras
G
Dicer-floxed-Kras
H
Dicer-wt-Kras
Dicer-het-Kras
Dicer-floxed-Kras
% of surface area
30
25
20
15
10
5
4
2
0
ADM
PanIN
Stroma

Figure 4.6: Kras-driven pancreatic lesions are sensitive to Dicer gene dosage.
(A-D) Brown staining indicates immunohistochemical labeling for Dicer (hemotoxylin counterstain, blue). (A) Wildtype pancreas. There is strong cytoplasmic labeling for Dicer in acinar cells. (B) *Dicer-wt-Kras*. Dicer is downregulated in acinar cells following oncogenic Kras activation. Notice that PanIN lesions (arrows) have higher Dicer expression. (C) *Dicer-floxed-Kras*. Residual nuclear signals likely represent non-specific antibody labeling. (D) In the *Dicer-floxed-Kras* pancreata, areas of ADM retain higher levels Dicer expression. Normal ductal epithelium, stromal cells and endocrine cells (lower right) also show normal expression of Dicer. E-G, H&E labeling demonstrates representative histology (E) *Dicer-wt-Kras*, (F) *Dicer-het-Kras* and (G) *Dicer-floxed-Kras* pancreata. (H) Quantification of surface area occupied by stromal infiltrate, ADM and PanIN. n>5 for each genotype. Error bars indicate standard error.

4.4 Discussion

Dicer is a master regulator of the miRNA pathway. The deletion of Dicer leads to loss of mature miRNAs (Chen et al., 2008a; Murchison et al., 2005). To assess the requirement of miRNAs in adult acinar tissue, we combined a floxed *Dicer* allele with an exocrine pancreas specific, tamoxifen-inducible Cre driver, *Mist1*^{CreERT2} (Habbe et al., 2008). In this report we show that *Dicer* is essential for the maintenance of normal acinar cell identity, with homozygous Dicer deletion resulting in loss of acinar cell polarity, and the induction of both ADM and EMT. When combined with activation of oncogenic Kras, homozygous Dicer deletion leads to accelerated ADM formation but no change in the rate of PanIN initiation or progression. We also present evidence that PanIN lesions select against complete loss of Dicer, and that Dicer haploinsufficiency accelerates PanIN initiation.

In non-neoplastic pancreas, histologic changes associated with conditional Dicer deletion were manifested within 72 hours following tamoxifen injection (data not shown). These cellular responses included loss of cell polarity and the induction of EMT and ADM, indicating an overall increase in cellular plasticity. *In vitro* induction of Dicer deletion in isolated pancreatic acini confirmed that these changes represented a cell autonomous effect of *Dicer* loss. In the *in vivo* setting, we did observe a robust inflammatory response after *Dicer* deletion (Fig. 4.1). In fact, some of the hyperplastic ductal-like structure we observed were not of apparent acinar cell origin, based on the absence of a heritable lineage marker (Fig. S4.1). While we cannot entirely rule out the possibility that the absence of lineage

marker expression is due to mosaic Cre activity, these structures may have arisen from ductal or other cell populations as a non-cell autonomous response to acinar cell injury.

In the absence of Kras activation, acinar cells eventually regenerated following widespread *Dicer* deletion. The source of newly generated acinar cells may similarly involve either proliferation of preexisting acinar cells that escaped *Dicer* deletion (Desai et al., 2007; Strobel et al., 2007), or from the expansion of a transdifferentiated/progenitor cell population such as ductal cells (Criscimanna et al., 2011).

When accompanied by Kras activation, ADM lesions persisted and progressed. Based on immunohistochemical labeling, these ADM and PanIN lesions seemed to select against complete loss of *Dicer*. This observation is in line with multiple cancer models in which enforced *Dicer* deletion cause inhibition of tumorigenesis, and tumors from *Dicer*$^{fl/fl}$ animals typically maintain one functional *Dicer* allele (Kumar et al., 2009; Lambertz et al., 2010; Yoshikawa et al., 2013; Zhang et al., 2013). One potential limitation of our study is that both the floxed *Dicer* alleles and *LSL-Kras*^{G12D} allele were recombined simultaneously in response to tamoxifen administration. One possibility is that during different stages of pancreatic tumor initiation and progression, there are divergent requirements for different levels of Dicer activity. Further studies that temporally separate the *Dicer* deletion and *Kras* activation events, as might be achieved using a tetracycline-regulated system (Furth et al., 1994; Saborowski et al., 2014), are needed to further investigate time- and tumor stage-dependent influences of Dicer.

A recent similar study by Morris et al was published describing the role of Dicer in the regulation of Kras-mediated ADM and PanIN (Morris et al., 2014). This study

reported that loss of *Dicer* compromised acinar identity and promoted Kras-driven ADM. At the same time, *Dicer* loss did not accelerate PanIN or PDAC development. Overall, their findings are comparable to our results. The major differences between the Morris paper and the current study involve the use of different Cre driver lines. In the study of Morris and colleagues, a *Pdx1:cre* line was utilized in which Cre activity is initiated in multi-lineage embryonic pancreatic progenitors at E12 (Heiser et al., 2006). In their setting, *Dicer* is conditionally deleted in all pancreatic epithelial lineages, while in our study, we have interrogated the function of *Dicer* specifically in adult acinar cells. Using this approach, we demonstrate that *Dicer* loss restricted to adult acinar cells is sufficient to induce an ADM reprogramming process, as characterized by the induction of DBA and Sox9 expression. A recent study demonstrated that the upregulation of Sox9 in the acinar population is an early tumor-initiating event (Kopp et al., 2012). Thus, miRNA deregulation in the acinar compartment may induce an early cellular change that renders acinar cells more prone to additional oncogenic stimuli, including Kras activation (Fig. 4.7).

One confounding factor in the current study is that there are different categories of miRNAs, some of them tumor suppressors, some of them oncogenes (Zhang et al., 2007). Yet Dicer knockout will impair the entire miRNA biogenesis pathway. In the future, we would like to pinpoint the role of individual miRNAs in pancreatic cancer initiation and progression. A good candidate is miR-217 (Zhao et al., 2010). In the next chapter, I would like to present our initial effort to employ zebrafish as a model to study pancreatic cancer and to address the role of miR-217 in pancreatic tumorigenesis.

In conclusion, we have established that Dicer is essential for the maintenance of acinar cell identity in the adult post-differentiated exocrine pancreas. Different levels of Dicer may be required at different stages of Kras-driven pancreatic tumorigenesis. Further work is needed to examine the functions of individual microRNAs and the time-dependent role of Dicer during pancreatic cancer initiation and progression.

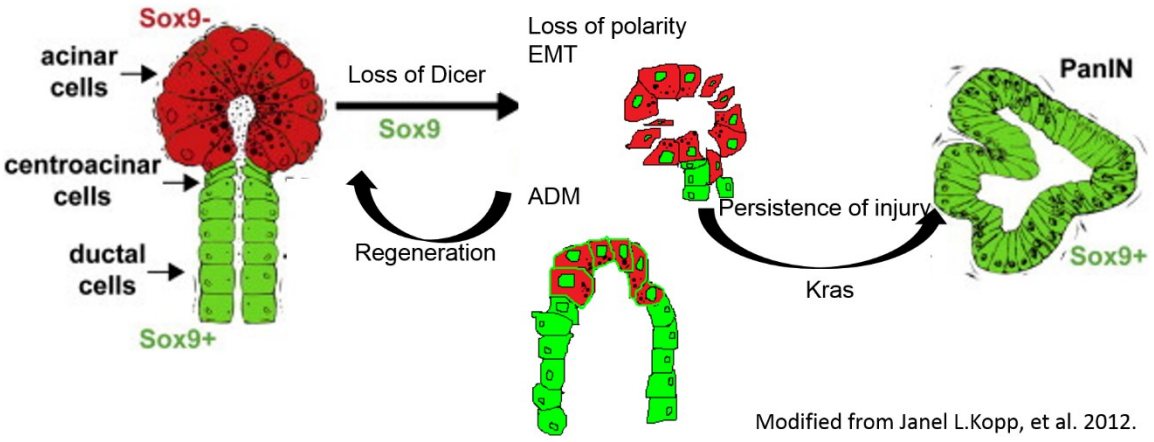
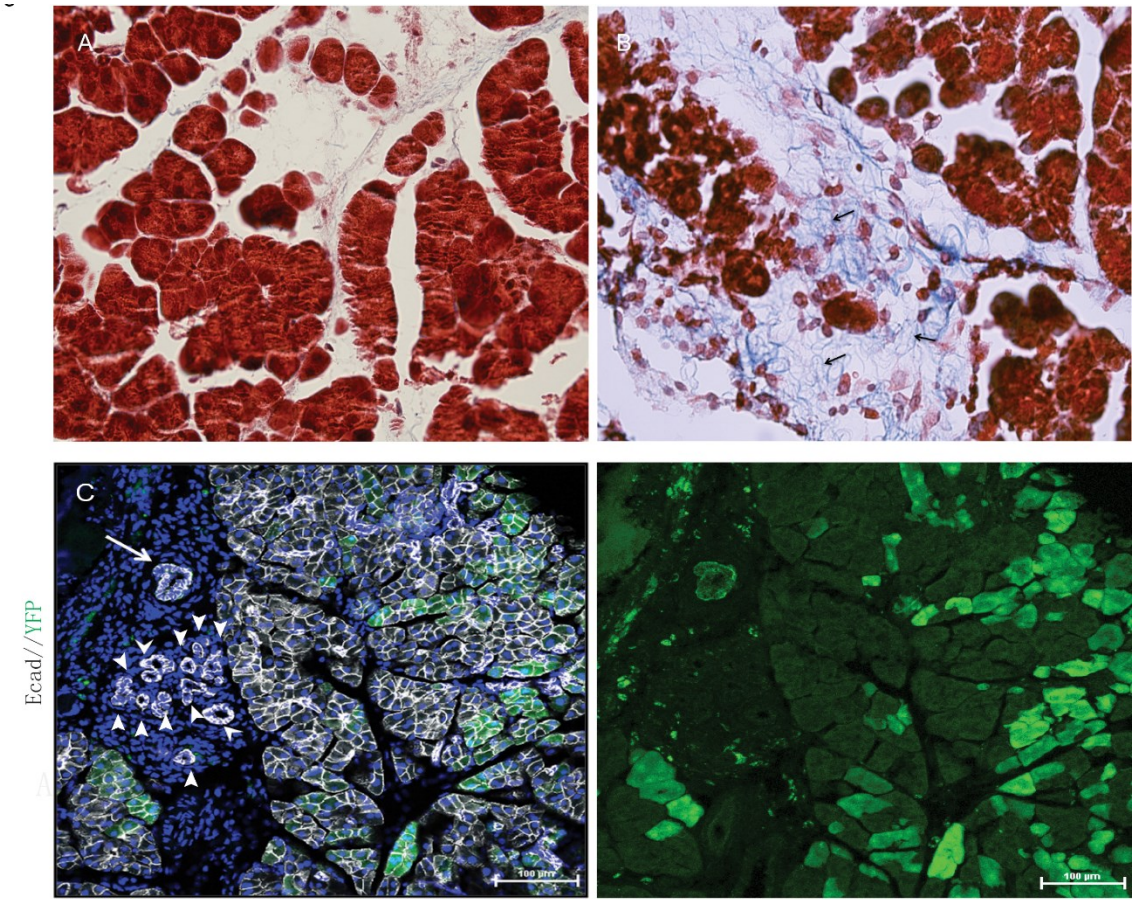


Figure 4.7: Summary of fate changes of acinar cells.
Temporary loss of *Dicer* induces cellular changes in the acinar population. These changes include loss of polarity, EMT and ADM. Acinar cells start to express ductal cell signature such as Sox9. Eventually, the pancreas regenerates. With the activation of oncogenic Kras, the injury persists in the acinar cells. This may eventually lead to cellular transformation and the ocurrence of early PanIN.


A
B
C
Ecad//YFP
100 µm
100 µm

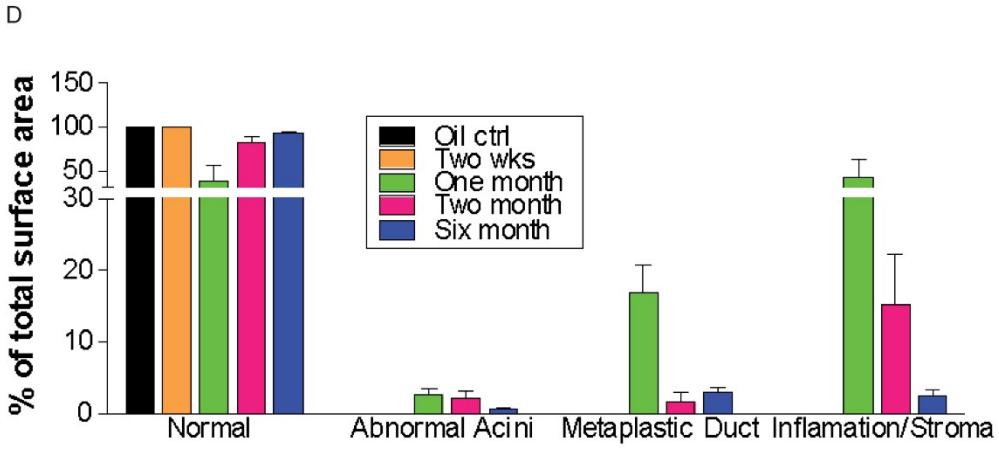

D
% of total surface area
150
100
50
30
20
10
0
Oil ctrl
Two wks
One month
Two month
Six month
Normal
Abnormal Acini
Metaplastic Duct
Inflamation/Stroma

Figure S4.1: *Dicer* deletion leads to pancreatic fibrosis.

(A-B) Trichrome staining shows fibrosis in the pancreas of 1 month post tamoxifen treatment. (A) Oil control. (B) 1 month post tamoxifen. There is severe phenotype with partial loss of acinar mass and sclerotic tissue. (C) 6 months after tamoxifen administration, YFP expression (green) becomes patchy. Some of the acinar units resemble ductal like structures as shown by Ecad (white) staining. Arrow points to one ductal like structure that is positive for YFP. Arrowheads point to several ductal like structures that are negative for YFP. (D) Progression of histologic changes following tamoxifen treatment. The most extensive changes are evident at one month post-tamoxifen. By 6 months, the pancreas is almost completely regenerated.

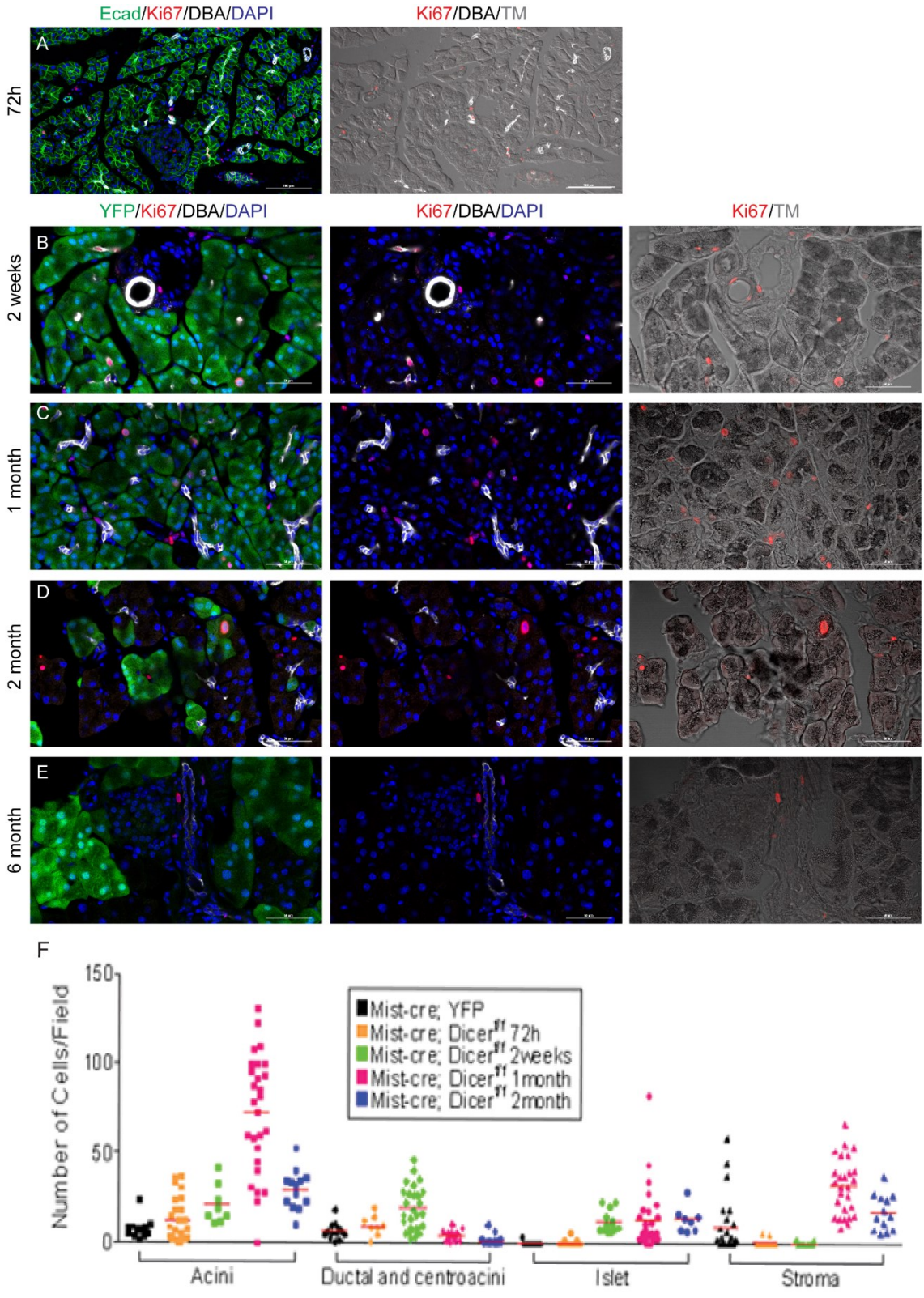

Ecad/Ki67/DBA/DAPI
Ki67/DBA/TM
A
72h
YFP/Ki67/DBA/DAPI
Ki67/DBA/DAPI
Ki67/TM
B
2 weeks
C
1 month
D
2 month
E
6 month
F
Number of Cells/Field
150
100
50
0
Mist-cre; YFP
Mist-cre; Dicer^ff 72h
Mist-cre; Dicer^ff 2weeks
Mist-cre; Dicer^ff 1month
Mist-cre; Dicer^ff 2month
Acini
Ductal and centroacini
Islet
Stroma

Figure S4.2: Tissue-wide proliferation response after *Dicer* deletion.
Ki67 (red) labels proliferating cells in pancreata (A) 75h, (B) 2 weeks, (C) 1 months, (D) 2 months, (E) 6 months post tamoxifen administration. In (A) Ecad is shown in green, DBA in white and DAPI in blue. In B-E, YFP (green) labels cell lineages in which Cre-mediated recombination has occurred. Note relative expansion of YFP-negative regions at 2 months and 6 months post-tamoxifen. Sections are co-stained with DBA (white) and DAPI (blue). Quantification of Ki67-positive cells per high power field is shown in (F).

Chapter 5. TALEN targeting the zebrafish mir-216b-217 miRNA cluster

5.1 Introduction

The zebrafish has evolved as an excellent model for cancer studying. It develops cancers that are histologically and genetically similar to human cancers. The ability to carry out *in vivo* live imaging, tumor transplantation, large-scale chemical and genetic screens, transgenesis, and with the advance of transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeat (CRISPRs), targeted knock-in and knock-out in zebrafish, offers a unique opportunity to study cancer biology and to functionally annotate the cancer genome in the post-genomic era (Feitsma and Cuppen, 2008; Liu and Leach, 2011b; White et al., 2013).

Pancreatic cancer is one of the most deadly cancers (American-Cancer-Society, 2014). Although progress has been made during the past few decades in the clinical management of pancreatic cancer patients, the 5 year survival rate for this cancer is still as low as 5% (American-Cancer-Society, 2014). Currently, the first line therapies for pancreatic cancer treatment are two chemotherapeutic agents, 5-fluorouracil and gemcitabine (Berlin et al., 2002; Burris et al., 1997; Neoptolemos et al., 2004). However, both of these drugs target DNA replicating events and thus are not cancer cell-type specific (Costello et al., 2012). It would be ideal if we can design an *in vivo* high throughput strategy to screen for potential drugs that specifically eliminate pancreatic cancer cells (Suggitt and Bibby, 2005). The other problem of pancreatic cancer is that there are few symptoms during its early stages. The majority of pancreatic patients present clinically with late stage metastatic cancer that is not surgically resectable (Li et al., 2004). Consequently, it is highly

relevant to generate animal models, either by transgenesis or xenograft, to better understand the pancreatic cancer biology and to discover early diagnostic markers (Hingorani et al., 2003; Kim et al., 2009a).

In our laboratory, we have been focusing on developing a zebrafish pancreatic cancer model. Our knowledge and experience from genetically engineered mouse models guides us in constructing zebrafish pancreatic cancer models. In human pancreatic cancer patients, the most frequently observed genetic alteration is activating mutation of Kras (Hruban et al., 1993; Jones et al., 2008). In parallel, the two most widely used mouse models for pancreatic cancer overexpress oncogenic Kras in a pancreatic specific manner. These two models are *Pdx1-Cre;LSL-Kras^{G12D}* and *Ptf1a-Cre;LSL-Kras^{G12D}* (Grippo and Tuveson, 2010; Herreros-Villanueva et al., 2012; Leach, 2004). Transgenic mice develop pancreatic lesions consistently and the cancer morphologies closely mimic the human counterparts (Hingorani et al., 2003). In our previous work, we established two zebrafish lines expressing oncogenic Kras (Liu and Leach, 2011a; Park et al., 2008b). In the first model, we modified a 160 kb BAC spanning the zebrafish *ptf1a* locus. The coding sequence of *ptf1a* was replaced with the human Kras^{G12V} cDNA (Park et al., 2008b). By nine months of age, the majority of transgenic fish developed discernible tumors. However, the fish carried predominantly acinar cell carcinomas as opposed to the most frequently observed duct-like structures in the human PDAC (Park et al., 2008b). Our second model utilized the Gal4/UAS system to express the human Kras^{G12V} in the exocrine pancreas (Liu and Leach, 2011a). Specifically, transgenic fish was created carrying *ptf1a:Gal4;UAS:eGFP-hKras^{G12V}*. However, this model also induced predominantly

acinar cell carcinomas with a few tumors containing mixed acinar and ductal differentiation (Liu and Leach, 2011a).

Using the newly generated *ptf1a:creER^{T2}* driver line (Chapter 2), we established a third transgenic fish line: *ptf1a:creER^{T2};ubi:loxp-CFP-loxp-Gal4;UAS:eGFP-hKras^{G12D}*. For the first time, we recovered fish that had developed PanINs histologically resembling human pancreatic lesions. Zebrafish PanINs were observed in 68.7% of the total pancreatic area and contained the spectrum of all histological grades (Joon Park, personal communication). Moreover, TALENs and CRISPRs made it feasible to carry out further targeted mutagenesis of potential tumor suppressors and oncogenes. Finally, we are equipped with all the right tools to functionally annotate the pancreatic cancer genome.

miR-217 is among the first candidates we set out to evaluate in the context of pancreatic cancer. It has been shown to function both as a tumor suppressor and an oncogene in different systems (de Yebenes et al., 2014; Kato et al., 2009; Su et al., 2014; Zhao et al., 2010). miR-217 is located within a miRNA cluster in the genome: mir-216b-217. This cluster has three miRNAs: miR-216b, miR-216a and miR-217. We targeted the zebrafish mir-216b-217 cluster using TALEN mutagenesis. We established independent founder fish lines carrying mutations for: (1) mir-216a and mir-216b; (2) mir-217; and, (3) deletions of the whole mir-216b-217 cluster. Homozygous mutant fish were hatched following Mendelian ratios; they were morphologically indistinguishable from wildtype controls. To evaluate the role of these miRNAs in pancreatic cancer, further crossing is in progress between these miRNA mutant lines and the *ptf1a:creER^{T2};ubi:loxp-CFP-loxp-Gal4;UAS:eGFP-hKras^{G12D}* line. Once we obtain fish with desired genotypes, we will

dissect out the fish pancreata at different time point following 4-Hydroxytamoxifen (4-OHT) injection and compare the cancer incidences as well as histological grades between miRNA knockout fish and miRNA wildtype fish.

Contributions: The following chapter represents the collaborative work of Dr. Joon Park and myself. Dr. Joon Park established the zebrafish pancreatic cancer model. He contributed Fig. 5.1. The rest of the work was fulfilled by myself.

5.2 Materials and Methods

5.2.1 TALEN mutagenesis

Please refer to Addgene Golden Gate TALEN assembly protocol, which is an expanded and slightly modified TAL assembly protocol published originally by Cermak, et al., Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting, Nucleic Acids Research, 2011, 39 (12) (Cermak et al., 2011).

The final destination vector used in our study is from David Grunwald lab: pCS2TAL3-DD and pCS2TALE3-RR (Dahlem et al., 2012) with restriction sites BamH1 and Sph1 flanking the inserts.

Correctly assembled pCS2TAL3-DD and pCS2TAL3-RR TALEN plasmids were linearized with Not1 and used as templates to generate 5′-capped mRNA by *in vitro* transcription (mMESSAGE mMACHINE SP6 kit, Life technologies, AM1340). About 150 pg of each mRNA was injected into the cytoplasm of 1 cell stage zebrafish embryos.

5.2.2 High-resolution melt analysis (HRMA)

HRMA was employed to assess the efficiency of TALEN-induced mutations in the injected F0 generation, or in the screening of germline transmission. HRMA was

performed according to manufacturer's instruction (Bio-Rad). Briefly, primers were designed to amplify an 85–125 bp amplicon that flanking the genomic target site. Care was taken in the designing of TALEN target sites so that the entire region was not polymorphic in the wildtype zebrafish. Precision melt supermix (Bio-Rad, 172-5110) was used to set up the following reaction:

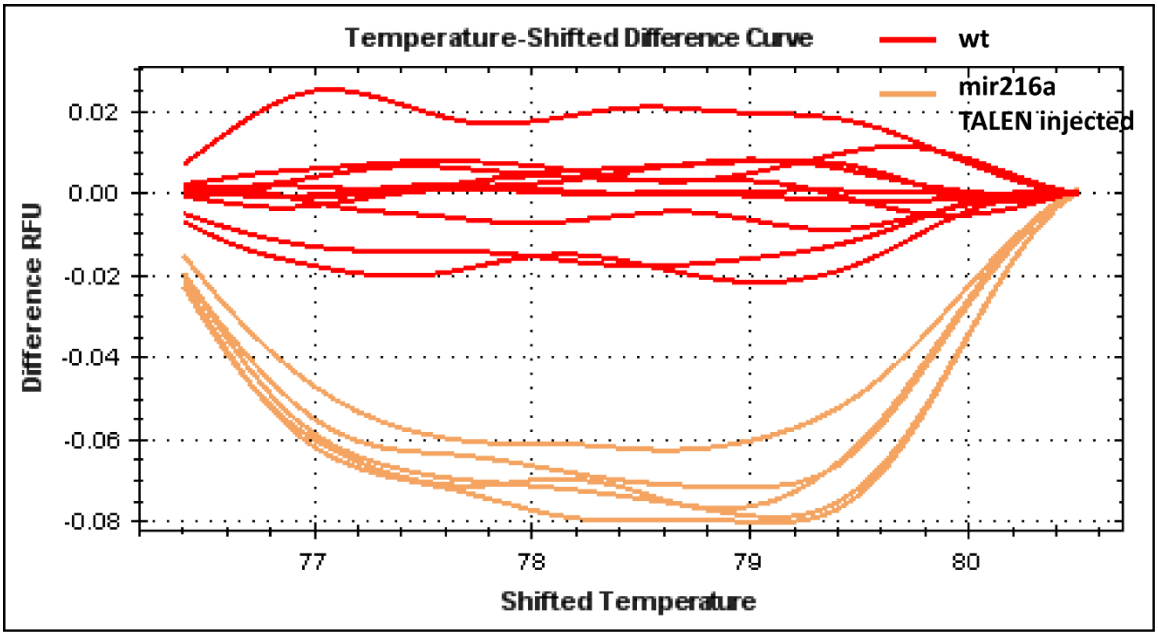
Component	Volume per 10 µl reaction, µl
Precision Melt supermix	5
2 µM (each) primer mix	1
DNase-free water	--
Genomic DNA	4
Total reaction mix volume	10

Amplification condition was: denaturation at 95 ºC, 2 min; 42 cycles of 95 ºC, 10 s; 58 ºC (or other primer-specific annealing temperature), 30 s; 72 ºC, 30 s. Melt curve was performed according the following procedure: denaturation at 95 ºC, 30 s; 60 ºC, 1 min; 65-95 ºC (in 0.2 ºC increments), 10 s/step.

Reaction was carried out on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Data were analyzed using Precision Melt Analysis™ software.

An example of HRMA result is shown below. Zebrafish embryos 2 days post TALEN injection were collected and pooled into groups of 5. These samples were subsequently incubated with 50 µl of DNA extraction solution prepared fresh (10 mM Tris,

pH 8.0, 10 mM EDTA, 200 μg/ml Proteinase K) for 3 hours at 50 ºC. After 95 ºC for 10 mins of heat inactivation, resulting DNAs were added directly to the HRMA reaction. HRM graph shows that the profiles from uninjected wildtype embryos clustered together. The TALEN injected embryos all clustered differently compared with wildtype, indicating the successful creation of indels.



5.2.3 Allele-specific PCR for genotyping

For offspring of established F1 generation, their genotypes were identified through allele-specific PCR. Primer sequences are listed in the following table.

Primer name	Sequences	Annealing temperature
mir-216a_b_M1	GACTGGGTAATCTCTGCAGGAT	58.5 ºC
	TTTGCAGGTGCTGTGTGAG	
mir-216a_b_M2	CTGGGTAATCTCTGCAGGCT	59.2 ºC
	AGGTGCTGTTTTGCAGGTG	
mir-216a_b_wt	CTGGGTAATCTCTGCAGGC	60 ºC
	AGGTGCTGTGTGTGACAGAA	
mir-217_M1	CATGAGAACTTTCTGATGTTGGA	49 ºC
	GCCTCTAGCGGGGAACTAAC	
mir-217_M2	GAGAACTTTCTGATGTTGGTGAATC	58 ºC
	GCCTCTAGCGGGGAACTAAC	
mir-217_M3	GAGAACTTTCTGATGTTGGTGATCA	58 ºC
	GCCTCTAGCGGGGAACTAAC	
mir-217_wt	TGGTGATACTGCATCAGGAACTG	62 ºC
	GCCTCTAGCGGGGAACTAAC	
HRM_mir-216b_F	GCATCACAGCCAAACTGCTA	54 ºC
HRM_mir-217_R	CTTTCGATGCTGATGGGAGT	

5.2.4 Generation of transgenic zebrafish

All experiments involving zebrafish were approved by the Johns Hopkins University Institutional Animal Care and Use Committee. Fish were raised and maintained under standard laboratory conditions. The following strains were established and/or utilized: *ptf1a:creER^{T2};ubi:loxP-eCFP-loxP-nuclear-mCherry*, *ptf1a:creER^{T2};ubi:lox-nuclear-eCFP-lox-Gal4-VP16*;*UAS:eGFP-KRAS^{G12D}*. Larvae were anaesthetized in 0.16% tricaine (3-aminobenzoic acid ethylester, A-5040, Sigma, pH 7.0). Adult zebrafish were euthanized by induction of tricaine anesthesia followed by placement in an ice bath, consistent with recommendations of the Panel on Euthanasia of the American Veterinary Association.

5.3 Results

5.3.1 Development of PanIN in the *ptf1a:creER^{T2};ubi:lox-nuclear-eCFP-lox-Gal4*;*UAS:eGFP-Kras^{G12D}* fish

The previous two fish lines we created were *ptf1a:Kras^{G12V}* and *ptf1a:Gal4;UAS:eGFP-hKras^{G12V}* (Liu and Leach, 2011a; Liu and Leach, 2011b; Park et al., 2008a). In terms of modeling pancreatic ductal adenocarcinoma (PDAC), the dominant form of human pancreatic cancer (Hezel et al., 2006), both of these lines have problems because they give rise to predominantly acinar cell carcinoma. We reasoned that the obstacle could have been the dependency of the expression of the oncogenic Kras on the *ptf1a* promoter, as *ptf1a* expression is restricted to acinar cells in the adult mature pancreas. Acinar ductal metaplastic transdifferentiation during pancreatic tumorigenesis may down-regulate the expression of *ptf1a* and subsequently compromise the expression of Kras. Thus

dysplastic acinar cells are selected against and tumor cells maintain their acinar identify (Means et al., 2005; Rodolosse et al., 2004). Coincidently, the mouse pancreatic cancer model, where *Ptf1a:cre* drives the oncogenic Kras expression, is in effect heterozygous for *Ptf1a*, due to the fact that the *Ptf1a:cre* allele in mouse is a knockin allele and the endogenous expression of *Ptf1a* from the same locus is prevented (Hingorani et al., 2003). Insights that we acquired from zebrafish *ptf1a* lineage tracing studies (Chapter 2) indicate that acinar cells with reduced *ptf1a* dosage have more plasticity. Consequently, haploinsufficiency in the mouse model of pancreatic cancer may be compatible with the observed ductal metaplasia.

To release Kras from complete *ptf1a*-dependency, we designed a third zebrafish pancreatic cancer model: *ptf1a:creER^{T2};ubi:lox-nuclear-eCFP-lox-Gal4*;*UAS:eGFP-Kras^{G12D}* (Fig. 5.1). The bipartite Gal4/UAS transgenic expression system was first developed in *Drosophila* and has since been adapted to zebrafish (Duffy, 2002; Halpern et al., 2008). In the activator line the transcriptional activator Gal4 is placed under the control of a specific promoter, while in the effector line the gene of interest is fused to the upstream activating sequences (UAS), which contains the DNA-binding motif of Gal4 (Scheer and Campos-Ortega, 1999). This system shows great sensitivity and strong signal amplification capability (Asakawa and Kawakami, 2008; Halpern et al., 2008). In our model, the expression of Gal4 is initially dependent on the *ptf1a* promoter activity, but subsequently the *ubiquitin* promoter will drive the continuous expression of Gal4 even if *ptf1a* levels are reduced. We were gratified to observe, for the first time, PanIN lesions in transgenic fish that morphologically resemble the human PanINs (Fig. 5.1).

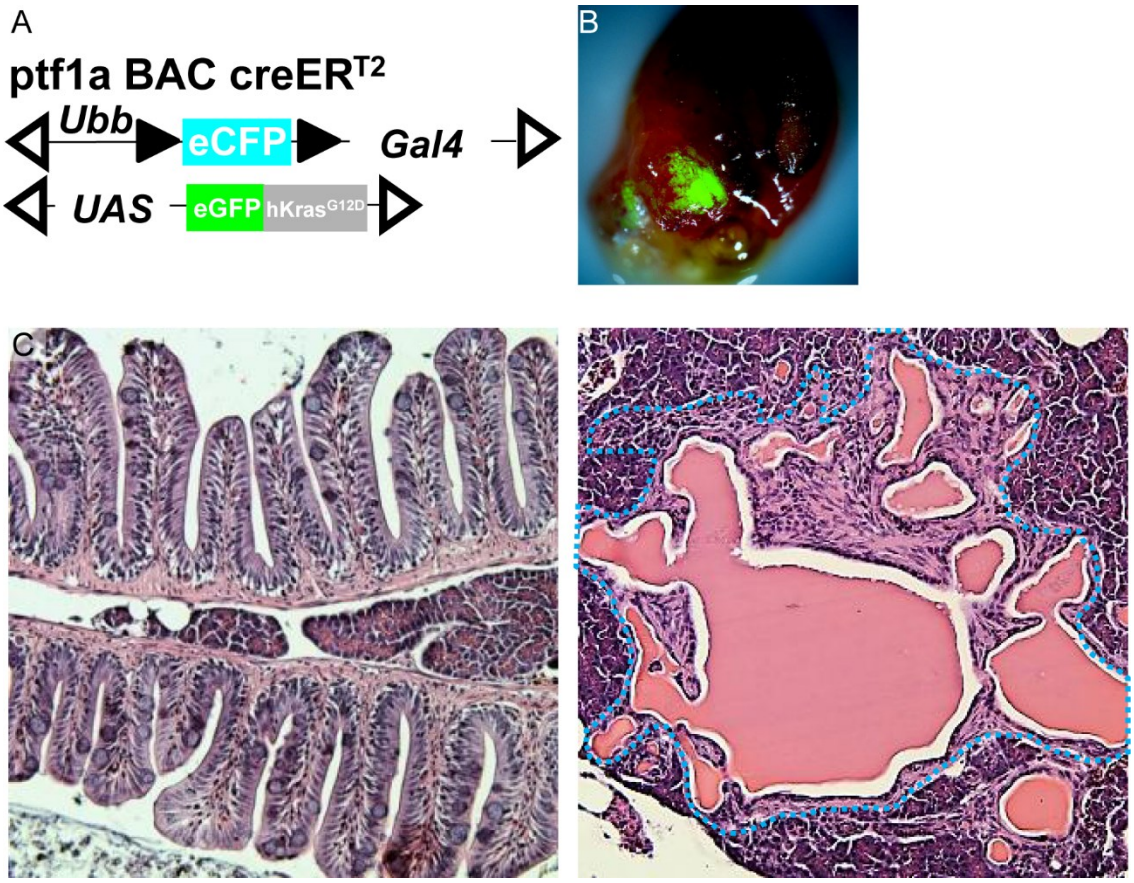


Figure 5.1: zebrafish pancreatic cancer model (unpublished data from Dr. Joon Park).

(A) The transgenic system we developed to induce zebrafish pancreatic cancer. (B) 24 weeks after 4-OHT injection, the gut bulb was dissected out. A tumor mass is discernable by eGFP fluorescence. (C) H&E from control fish. (D) H&E from transgenic fish 24 weeks after 4-OHT treatment. PanIN like lesions are observed (dashed line).

5.3.2 The mir-216b-217 miRNA cluster

Having developed a zebrafish model of pancreatic cancer that recapitulates the human pathology, we proceeded to employ this system to test oncogenic/tumor suppressor activities of different genes and genomic elements.

One interesting candidate is miR-217. miR-217 has been shown to function as a tumor suppressor and targets Kras in pancreatic cancer (Zhao et al., 2010). Overexpression of miR-217 inhibits the anchorage-independent growth of pancreatic cancer cell lines and reduces tumor load in a xenograft model (Zhao et al., 2010). In human, miR-217 is down regulated in PDAC and PanIN but not in pancreatitis, establishing it as a potential biomarker in diagnostic procedures (Ali et al., 2012; Schultz et al., 2012; Szafranska et al., 2007; Xue et al., 2013; Zhao et al., 2010).

In the zebrafish genome, miR-217 is located in a cluster along with miR-216a and miR-216b within a 600 bp section on chromosome 22 (Fig. 5.2A). Individual miRNAs in the cluster are among the most evolutionarily conserved miRNAs (Kiezun et al., 2012). In addition, the cluster configuration is preserved. In the zebrafish, all three miRNAs seem to be transcribed from one polycistron (Fig. 5.2A', A''). They are expressed specifically in the pancreatic acinar cells during development and in adult (Wienholds et al., 2005) (Fig. 5.2B-G).

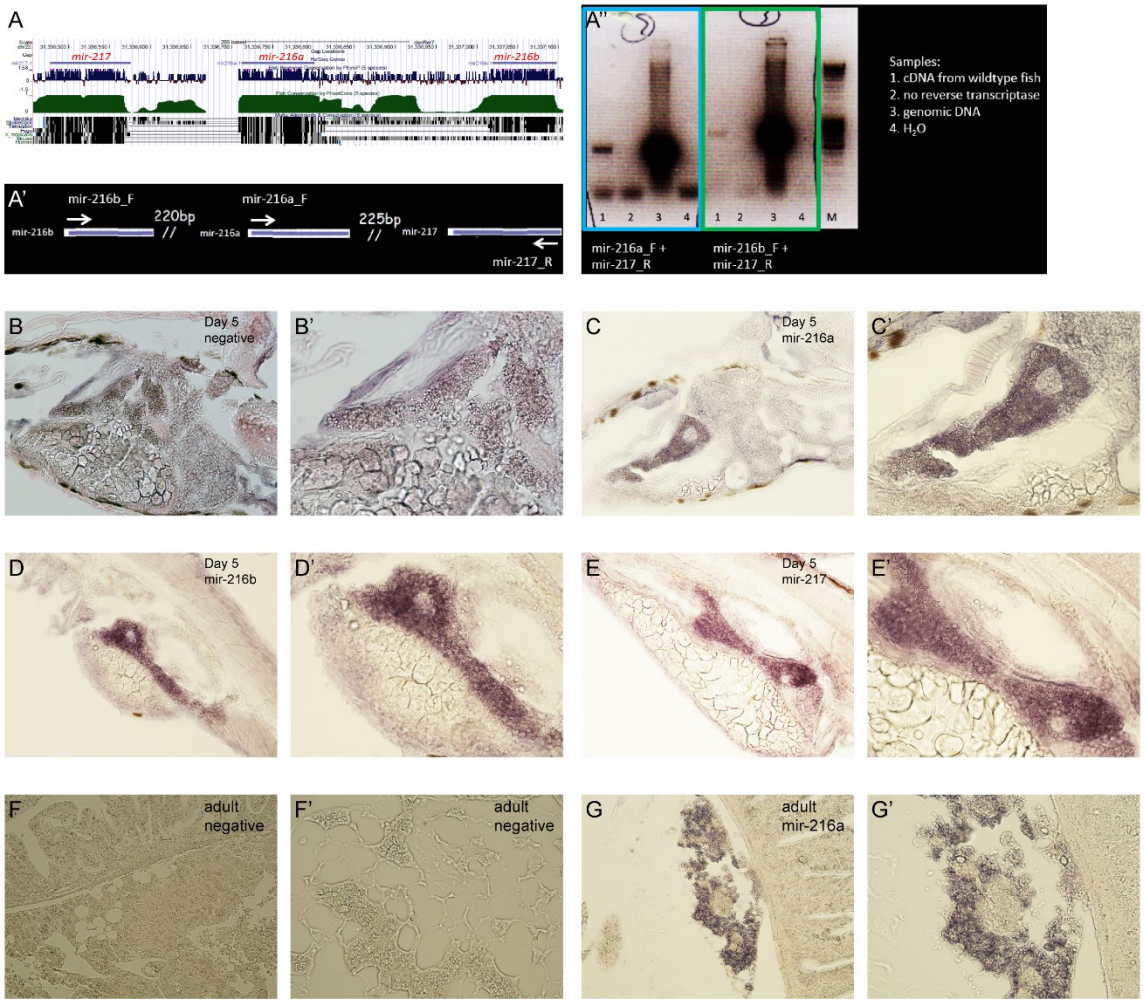


Figure 5.2: mir-216b-217 genomic locus and expression pattern.

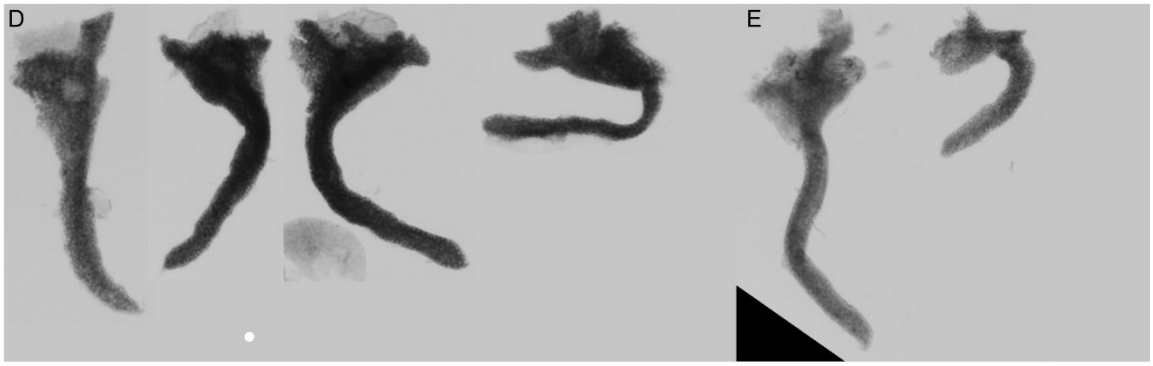
(A) UCSC genome browser screen-shot shows the relative location of the three miRNAs. They are located on the reverse strand of Chr.22. A', locations of primers used in RT-PCR reaction. For clarity, the mir-216b-217 cluster is shown with the transcription starting site on the left. A'', agarose gel showing products from RT-PCR reaction. Primers used in the reaction are indicated at the bottom of the gel. Templates are listed on the right. The faint band in lane 1 with mir-216b_F + mir-217_R primer pair indicates that the whole cluster is co-transcribed. (B-G) LNA *in situ* hybridizations of miRNAs. (B-E) Sections from 5 dpf fish. (F-G) Section from adult fish. *In situ* hybridization was developed using NBP/BCIP reagents and signals are purple-blue. All the miRNAs have acinar specific expression. Probe used for labelling is shown on the right corner on each micrograph.

5.3.3 Targeting the mir-216b-217 miRNA cluster using TALEN

We made use of the powerful Transcription activator-like (TAL) effector nucleases (TALENs) system to create mutations in the mir-216b-217 microRNA cluster. TALENs have been used to introduce locus-specific double-stranded breaks as well as homology-derived repairs with little off-target effects in zebrafish (Bedell et al., 2012; Dahlem et al., 2012). We designed 3 pairs of TALENs targeting mir-216a, mir-216b and mir-217 individually. Taking into consideration of the potential functional redundancy of miRNAs within the same miRNA family (Brennecke et al., 2005), we decided to mutate mir-216a and mir-216b simultaneously, aiming for bi-locus mutagenesis. Furthermore, all the miRNAs within the miRNA cluster may have similar biological functions (Kim et al., 2009b). As a result, a deletion mutation encompassing the entire region is desirable to reveal the function of the miRNA cluster. The above mentioned schemes can be easily achieved by injecting zebrafish embryos with multiplexed TALEN pairs targeting various loci. We observed that, when injected individually, each TALEN pair achieved 100% efficiency in introducing targeted small indel mutations (assessed by HRMA). By comparison, when two pairs of TALENs were co-injected, the efficiency dropped. Approximately 50% of the resulting fish carried mutations in at least one loci. Furthermore, when two TALEN pairs are within short distance apart, instead of recovering mutants carrying deletions spanning the entire region, we obtained fish carrying small indels for each locus (Fig. 5.3A). However, when TALEN targets are located further apart, mutants with big deletions were obtained (Fig. 5.3C). Figure 5.3 summarizes the founder lines we created carrying mutations for different miRNAs. In summary, TALEN technology

facilitates targeting of multiple genomic regions simultaneously that may be challenging or time consuming to target by traditional methods. By co-injection of multiple TALEN pairs, multi-locus mutagenesis can be achieved in a single generation.


A
mir-216b
mir-216a
wt: TgactgggtaatctctgcaggcaactgtgatgtgattacagtctcacA……TctcacaggcgctgctgggttctgtcacacacagcacctgcaaaacA
M1: Tgactgggtaatctctgcagg-a--tgtgatgtgattacagtctcacA……Tctcacaggcgctgct----------cacacacagcacctgcaaaacA
M2: Tgactgggtaatctctgcagg---ctgtgatgtgattacagtctcacA……Tctcacaggcgctgctgggcacctg-caaa-acagcacctgcaaaacA
B
mir-217
wt: TgagaactttctgatgttggtgatactgcatcaggaactgattggatgatA
M1: Tgagaactttctgatg------------------------ttggatgatA
M2: Tgagaactttctgatgttggtga------atcaggaactgattggatgatA
M3: Tgagaactttctgatgttggtg-------atcaggaactgattggatgatA
C
mir-216b
mir-217
wt: TgactgggtaatctctgcaggcaactgtgatgtgattacagtctcacA……TgagaactttctgatgttggtgatactgcatcaggaactgattggatgatA
tgatgtga
M1: TgactgggtaatctctgcaggcaactgtgatgtgattacagtctcacA……Tgagaactttctgatgttggtgat-------caggaactgattggatgatA
M2: Tgactgggtaatctctgcag----------……----------gaactgattggatgatA
M3: Tgactgggtaatctctgca----------……----------atcaggaactgattggatgatA


D
E

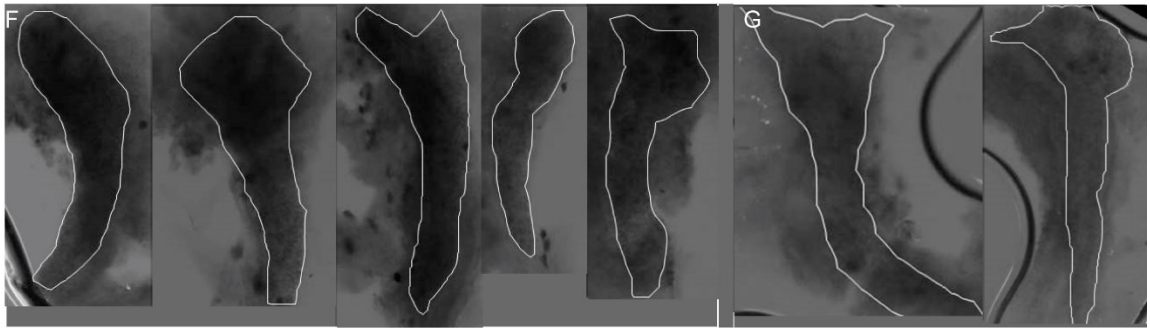

F
G

Figure 5.3: Establishing F1 founders carrying mutations in the mir-216b-217 miRNA cluster.

(A) Sequences of the founder lines carrying mutations in mir-216a and mir-216b region. (B) Sequences of the founder lines carrying mutations in mir-217 region. (C) Sequences of the founder lines carrying mutations in mir-216b-217 cluster. Notice that M1 founder fish has small indels in mir-216b and mir-217 independently, instead of carrying deletion of the whole cluster. In all the sequences, blue nucleotides indicate the TALEN target sites. Short hairpin structures of pre-miRNAs are shown above each individual sequences. Underlined nucleotides are part of the mature miRNAs. (D-G) miR-216a *in situ* hybridization on (D, F) d6 wt fish pancreata, or, (E, G) d6 mir-216b-217 homozygous mutant pancreata. (D, E) and (F, G) are two independent experiments. Hybridization Signals from the homozygous mutant fish are reduced. In (F, G), the pancreata are outlined.

5.3.4 Homozygous mutant fish of mir-216a/b, mir-217 and the mir-216b-217 miRNA cluster do not show developmental defects

We crossed heterozygous F1 founder fish with reporter fish lines carrying transgenes *ptf1a:GFP*, *ins:mCherry* and/or *Tp1:hmgb1-mCherry (THM)* to label acinar cells, endocrine β cells and Notch-responsive cells, respectively. Resulting fish were intercrossed to derive fish homozygous for each mutation. At 6dpf, we dissected out the pancreata and checked for marker expressions. Homozygous mutant fish of mir-216a/b, mir-217 or mir-216b-217 were hatched according to Mendelian ratios and did not manifest major developmental defects (Fig. 5.4).

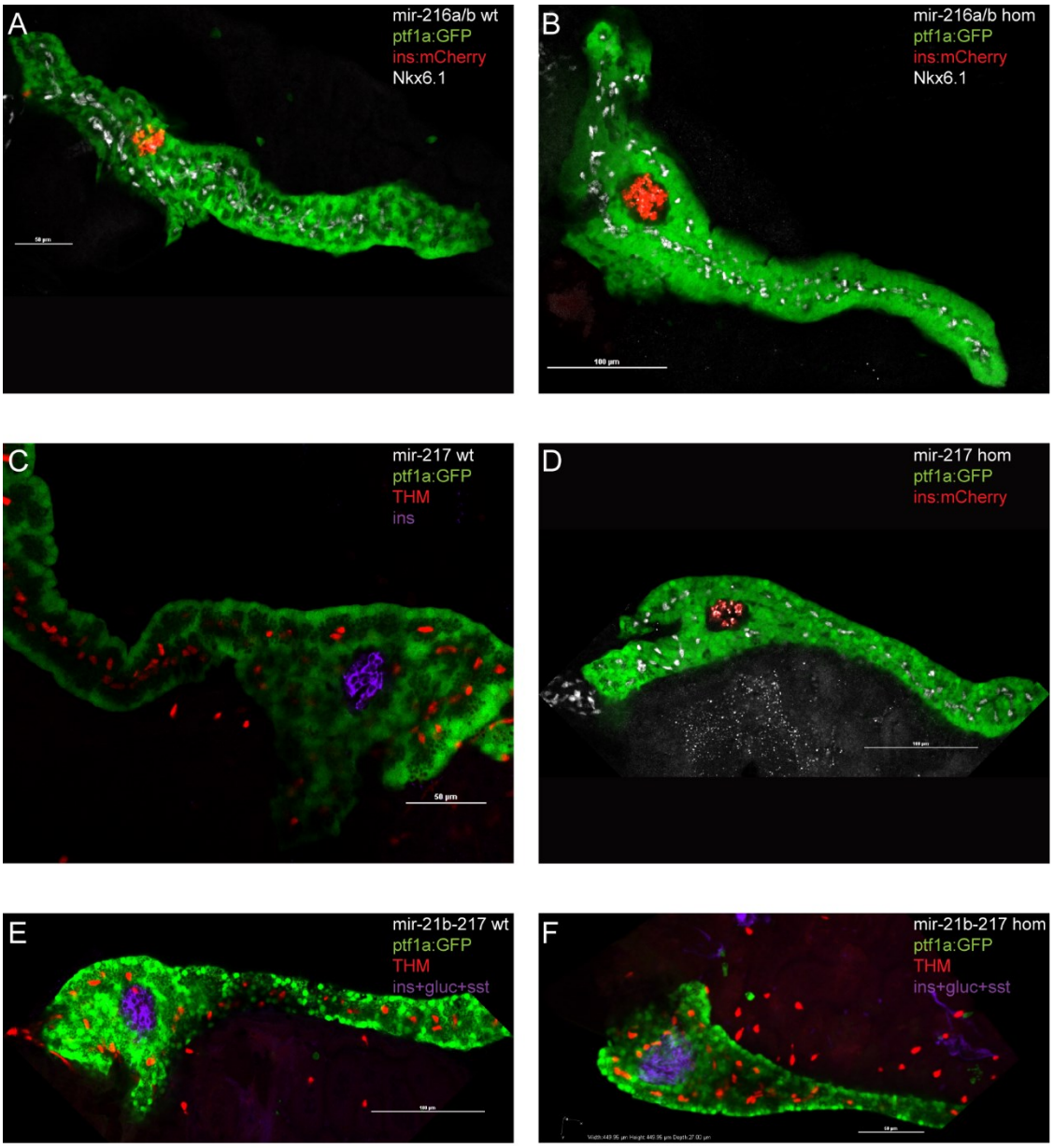


Figure 5.4: Homozygous mutant fish for each miRNAs do not display developmental defects.

Exocrine pancreas is labeled by the ptf1a:GFP transgenic marker. Endocrine components are indicated by ins:mCherry transgene or by immunofluorescent staining for Insulin (Ins), Glucagon (Gluc), Somatostatin (Sst). Pancreatic Notch-responsive cells are labeled by the transgene THM or by immunofluorescent staining for Nkx6.1.

5.3.5 The role of mir-216a, mir-216b and mir-217 in pancreatic cancer initiation and progression

Having generated the corresponding mutations, we now propose to make use of the newly generated zebrafish pancreatic cancer system to address the role of these miRNAs in pancreatic cancer initiation and progression. Fig. 5.5 shows our crossing scheme to generate fish with all the transgenic and mutation components.

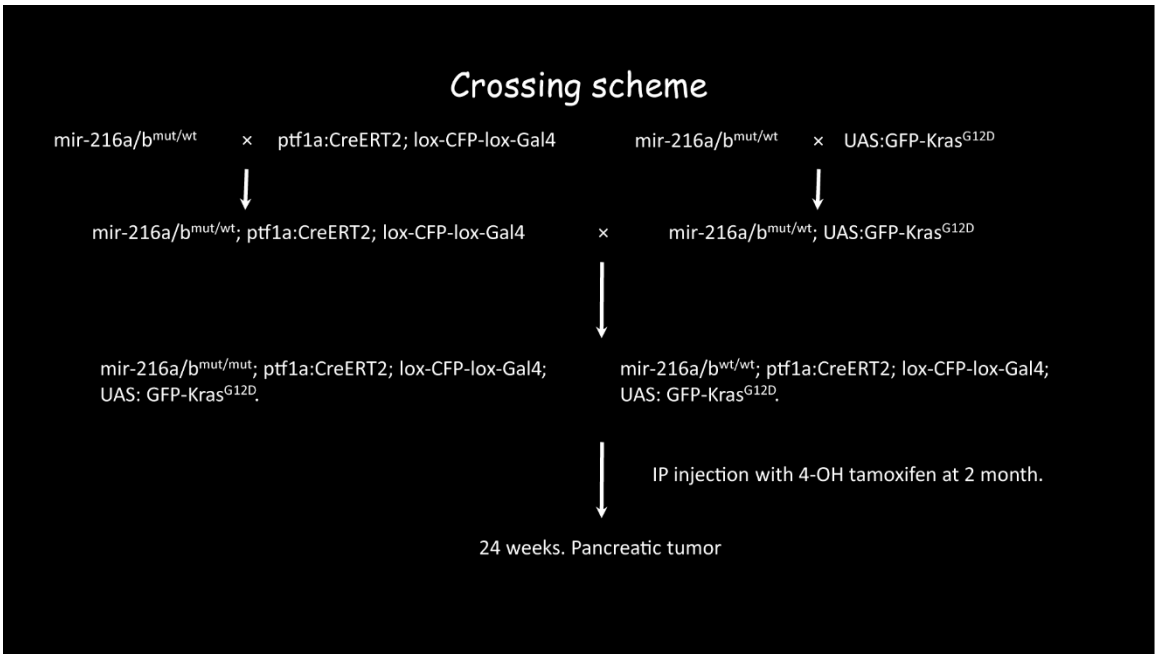


Figure 5.5: Crossing scheme.

The current experimentation represents our initial effort to assess the pipeline to functionally annotate the pancreatic cancer genome in zebrafish. Once the system is set up, we will move on to create more zebrafish mutant lines carrying orthologs of mutations having been discovered in human pancreatic cancer. Especially, the large-scale targeted genome-editing approaches made available by TALEN and CRISPR technologies broaden our inquiry not only to tumor suppressors but specific oncogenic mutations as well.

5.4 Conclusion

In summary, we created a zebrafish pancreatic cancer model that recapitulates the human pancreatic cancer histology. Using the newly developed exciting TALEN technique, we created zebrafish carrying mutations of potential tumor suppressors. No apparent developmental defects were observed in homozygous mutant fish. Currently, crosses of different transgenic fish are on-going in order to generate the fish with desired genetypes. They are: *mir-216a/b*$^{mut/mut}$*;ptf1a:creER*^{T2}*;lox-eCFP-lox-Gal4;UAS:Kras*^{G12D}, *mir-217*$^{mut/mut}$*;ptf1a:creER*^{T2}*;lox-CFP-lox-Gal4;UAS:Kras*^{G12D}, and *mir-216b-217*$^{mut/mut}$*;ptf1a:creER*^{T2}*;lox-CFP-lox-Gal4;UAS:Kras*^{G12D}. We are in the final stage of developing an *in vivo* testing pipeline to functionally annotate the pancreatic cancer genome.

There are still some potential caveats of the system that still need to be addressed. First of all, the penetrance of pancreatic cancer is low in our *ptf1a:creER*^{T2}*;ubi:lox-CFP-lox-Gal4*;*UAS:eGFP-Kras*^{G12D} model. Preliminary data showed that around 11% of the fish carrying all the genetic components would develop PanIN and pancreatic tumor (Dr. Joon Park, personal communication). The reason of the low penetrance is likely to be the reduced efficiency of *ptf1a:creER*^{T2} activity in the adult fish. For those fish in which we did not observe tumor mass, eGFP activity was not detected as well. Furthermore, in the *ptf1a:creER*^{T2}*; ubi:loxp-CFP-loxp-nuclear-mCherry* control fish, adult fish have similarly low Cre activity. We will need to screen for the best fish line that exhibits high inducible Cre activity in adult and expand them so as to reduce the variability of results among

different fish. Second, the average time it takes to develop PanIN lesions in zebrafish is more than 24 weeks; whereas in the mouse model of pancreatic cancer, lesions with similar histological grade starts to appear as early as 3 weeks. We can potentially shorten the time course of cancer development in zebrafish by cerulein injection to induce pancreatitis (Carriere et al., 2009; Guerra et al., 2007), or alternatively, by increasing the copy number of the *UAS:eGFP-KrasG12D* transgene through plasmid injection. Park et al. recently showed that injection of *UAS:eGFP-KrasG12D* plasmid into *ptf1a:Gal4* fish can induce pancreatic tumors by 3 months of age. (Park et al., 2014).

Chapter 6. Conclusions

6.1 Research summaries

In summary, during my Ph.D. training, I explored different aspects of the pancreatic biology.

In Chapter 2, I described the lineage analysis of the *ptf1a*-expressing cells during zebrafish development. I demonstrated that there are a small number of ptf1a-expressing cells giving rise to pancreatic Notch-responsive-cells (PNCs) and endocrine cells during development. The *ptf1a* lineage to some degree contributes to the regeneration of β cells. In the *ptf1a* heterozygous mutant fish, a higher proportion of *ptf1a* lineage-labeled cells are traced into PNCs and endocrine compartment. Further reduction of ptf1a dosage converts the ventrally derived pancreatic progenitor cells to gall bladder and other nonpancreatic cell fate.

In Chapter 3, I presented the characterization of a potential progenitor population, A+Sca-1+ cells, in the adult mouse pancreas. A+Sca-1+ cells have mesenchymal stem cell features. They have the capacity to be differentiated into both endocrine and exocrine cells in the dorsal bud *ex vivo* culture system. Furthermore, this population of cells secrete signaling molecules that highly resemble those secreted by embryonic mesenchyme.

In Chapter 4, I showed the work we performed on deciphering the role of *Dicer* in the mature acinar cells during normal tissue homeostasis and pancreatic cancer initiation. We found that *Dicer* is essential for the maintenance of acinar cell identity. Acinar cells lacking *Dicer* showed increased plasticity, as evidenced by loss of polarity, initiation of epithelial-to-mesenchymal transition (EMT) and acinar-to-ductal metaplasia (ADM). In

the context of oncogenic Kras activation, the initiation of ADM and pancreatic intraepithelial neoplasia (PanIN) were both highly sensitive to *Dicer* gene dosage. Homozygous *Dicer* deletion accelerated the formation of ADM but not PanIN. In contrast, heterozygous *Dicer* deletion accelerated PanIN initiation, revealing complex roles for *Dicer* in the regulation of both normal and neoplastic pancreatic epithelial identity.

Finally, in Chapter 5, I outlined our effort to establish the zebrafish model of pancreatic cancer and our initial attempt to annotate the pancreatic cancer genome *in vivo* by functional genomic approaches as typified by TALEN mutagenesis.

The different parts of my thesis work are interconnected. In general, adult tissue-resident stem cells are believed to be allocated during early development (Barker et al., 2010). These somatic stem cells maintain tissue function by replacing terminally differentiated, aged or damaged cells (Mimeault and Batra, 2008). It is widely thought that cancers originate from these preexisting stem cells and the tumorigenesis process "borrows" the developmental programs and "hijacks" physiological homeostasis mechanisms (Lobo et al., 2007; Reya et al., 2001). By studying the entire process, from development to adult stem cells to cancer, we can gain extensive knowledge of the regulatory pathways of normal biological system as well as cancer ontogeny.

One common theme emerged from my studies is the plasticity of pancreatic cells. Example 1: During development, I showed that cells with reduced dosages of *ptf1a* are more inclined to differentiate into pancreatic PNCs and endocrine cells. Example 2: In the adult pancreas, I identified an A+Sca-1+ population that can be differentiated into multiple mesenchymal lineages *in vitro* and endocrine/exocrine cell fate in dorsal buds explant *ex*

vivo. Example 3: Adult pancreatic acinar cells undergo epithelial mesenchymal transition and acinar ductal metaplasia upon *Dicer* deletion. The cellular plasticity is a double-edged sword. On one hand, the ease of cell fate conversion makes it conceivable to design a strategy to convert different cell types in the pancreas into insulin-secreting β cells. Both type 1 and type 2 diabetes patients suffer from β-cell deficiency. They will both benefit from the increase of β-cell numbers. On the other hand, the transdifferentiation of acinar cells may be the underlying mechanism of pancreatic neoplastic transformation. Theoretically, if we devise a way to maintain the acinar cell fate or reverse the cell-fate conversion process, we may be able to improve treatment of pancreatic cancer patients.

The other valuable insight we gained from my thesis work is that there are striking similarities in the regulatory mechanisms between mouse and zebrafish. Our observations, together with accumulated knowledge, show that: the pancreas derives from dorsal and ventral anlagen in both species; *Ptf1a* is one of the earliest transcription factors expressed in the pancreatic field; reduced dosage of *Ptf1a* is correlated with greater lineage differentiation potentials; progenitor cells with severe reduction of *Ptf1a* levels transfated into gall bladder or other cell types. Furthermore, oncogenic Kras mediates pancreatic tumorigenesis stemming from the acinar population in both mouse and zebrafish.

Traditionally, mouse has been the favorite model of modern genetics due to the availability of embryonic stem cells, homologous recombination and other genetic engineering technologies. For zebrafish, although there are strategies such as random mutagenesis using ENU, retroviruses or transposons, the ability to engineer specific mutations has remained elusive. However, the latest introduction of TALEN and CRISPR

methods for targeted genomic editing makes it possible to disrupt or modify almost any locus of interest in an ever-expanding array of organisms (Gaj et al., 2013). We now have the ability to carry out the majority of genetic manipulations equally well in both mouse and zebrafish. There are great benefits of working with both species simultaneously. Mouse is an ideal organism for modeling human disease because they more closely resemble human in terms of underlying physiology and genomic organization. In addition, extensive resources, such as International Knockout Mouse Consortium (IKMC), Knockout Mouse Project Repository (KOMP), Allen Brain Atlas, and GenePaint, already exist as a result of collaborative efforts to facilitate functional studies in mice. On the other hand, zebrafish is an ideal organism for early developmental studies and high throughput screening because of their optical transparency, big clutch size and rapid *ex vivo* development. By comparing and contrasting between zebrafish and mouse, we can more readily obtain insightful understanding of the universal biological principles.

Diabetes and pancreatic cancer are two devastating diseases of the pancreas. My thesis work is on the basic science aspects of the pancreas; but hopefully, it will provide some incremental knowledge to help bridge the gap from benchside to bedside. There are several points I would like to emphasize. First, the lineage tracing work demonstrates that a reduced level of *ptf1a* is more conducive to β cells differentiation. This may guide us in designing a more efficient protocol for the derivation of β cells *in vitro*. Second, in the adult pancreas, A+Sca-1+ cells have great potentials in both serving as a source for new β cells and providing a supportive niche for proper cellular differentiation. Third, the level of Dicer is tightly regulated during the initiation of pancreatic cancer. The oncogenic as well

as tumor suppressor roles of Dicer seems to be context-dependent and warrants further intensive research to evaluate its feasibility as a therapeutic target. My thesis work is definitely not conclusive and a lot more work is needed to carry each project further. Nevertheless, I hope it has provided some insight into the pancreatic biology. May we one day find the cure for both diabetes and pancreatic cancer.

6.2 Limitations and Future directions

There are several limitations in my work.

In Chapter 2, we utilized the *ptf1a:creER^{T2};ubi:loxp-CFP-loxp-nuclear-mCherry* double transgenic fish to map the lineage of *ptf1a*. We did not observe large contributions of the early *ptf1a*-expressing cells to endocrine nor PNCs. However, negative results from this lineage tracing experiment could have been explained by numerous factors rather than true noncontribution (Kawaguchi et al., 2011). In our case, potential issues include low efficiency of labelling; inappropriate timing of 4-OHT administration; and slow kinetics of Cre. Moreover, the *ptf1a:creER^{T2}* allele is carried by a randomly integrated BAC transgene. It may not retain all the regulatory elements of the native *ptf1a* genomic locus or suffer from position effects (Wilson et al., 1990). As a result, it may not reliably follow the endogenous expression pattern. In addition, we claimed that the Sanger mutant *ptf1a^{sa126}* was a null or hypomorphic allele, however, biochemical quantification of the dosage of functional Ptf1a is lacking. We may also take advantage of the other *ptf1a* hypomorphic mutant *akreas* line (Dong et al., 2008) to generate an assemblage of zebrafish lines containing different dosages of *ptf1a* to address the threshold effect of *ptf1a* in cell fate determination in detail.

In Chapter 3, we identified an A+Sca-1+ population that have progenitor cell properties. However, we did not have a lineage tracing strategy to map the fates of these cells *in vivo* and hence we are not clear of the endogenous functions of this population. Furthermore, we found that this population secretes a number of cytokines that were similarly secreted by embryonic pancreatic mesenchymes. But whether these cytokines play a role in paracrine signals and what the targets are remain to be addressed. Similarly, the upregulation of A+Sca-1+ population during acute pancreatitis was purely an observation. We do not have evidence of its functional significance.

In Chapter 4, we studied the role of *Dicer* in mature acinar cells using $Mist1^{CreERT2/+};Dicer^{fl/fl}$ transgenic mice. However, we did not know whether the effects of *Dicer* deletion were due to the depletion of miRNAs or through miRNA-independent *Dicer* mechanism. The observation that under conditions of oncogenic Kras activation, there was tissue-wide *Dicer* downregulation is interesting but we did not know the mechanism. At the same time, we did not have an accurate assessment of the extent of Dicer downregulation in the context of different genotypes or in different ADM and PanIN lesions. Also, we only examined the *Dicer* depletion phenotypes in the Kras activation condition by means of tissue histology, but we did not collect information of the overall survival status of the corresponding transgenic mice. Detailed miRNA microarray studies should also be carried out to monitor the expression profile of each individual miRNA.

In Chapter 5, we developed a zebrafish model of pancreatic cancer. However, the cancer incidence was only about 11% in contrast to the near 100% penetrance of in the corresponding mouse models of pancreatic cancer. In addition, the earliest time at which

we started to observe histological abnormalities in zebrafish was around 24 weeks. The problem of low penetrance and slow time course of our system need to be solved before the strategy can be used effectively in downstream applications such as high throughput drug screening and functional genomics. Furthermore, the study of the role of mir-216b-217 in pancreatic cancer initiation is currently not complete.

Everything considered, tissue specification and fate maintenance is a tightly controlled control process. This principle is exemplified in the pancreatic system we studied here.

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Biography

I was born in Beijing in April, 1985, the year of the ox. I was always very curious about the world and wanted to find answers to all the questions of life. I aspired to be a scientist at a very young age. I went to Tsinghua University, the best university in China, in 2003. I majored in Biological Science and Biotechnology. I obtained my Bachelor of Science degree in 2007, with a GPA of 4.0 (out of 4).

After graduation from Tsinghua University, I directly entered the Human Genetics program at Johns Hopkins to pursue my Ph.D. degree. I initially worked for Dr. Nicholas Katsanis on the collected group of diseases, ciliaopathy. In 2009, Dr. Katsanis left for Duke University. I subsequently joined Dr. Steven Leach's laboratory in January, 2010. The work in the Leach laboratory is on different aspects of pancreas biology, from development to pancreatic cancer. In January, 2014, Dr. Leach left for Memorial Sloan-Kettering. Since then, Dr. Michael Parsons has assumed the role of my thesis co-advisor. During the training as a graduate student, I authored or co-authored the following publications: (1) *Dicer is required for maintenance of adult pancreatic acinar cell identity and plays a role in Kras-driven pancreatic neoplasia; (2) Differential in vivo tumorigenicity of diverse KRAS mutations in vertebrate pancreas: A comprehensive survey; (3) A red-shifted fluorescent substrate for aldehyde dehydrogenase; (4) In Vitro Screening for Compounds That Enhance Human L1 Mobilization*; *(5) Sphere-forming assays for assessment of benign and malignant pancreatic stem cells; (6) Notch signaling regulates neuroepithelial stem cell maintenance and neuroblast formation in Drosophila optic lobe development; (7) A splice-site mutation in a retina-specific exon of BBS8 causes nonsyndromic retinitis pigmentosa.*